Dye Coupling between Pyramidal Neurons in Developing Rat Prefrontal and Frontal Cortex Is Reduced by Protein Kinase A Activation and Dopamine

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During early postnatal development, lamina II/III pyramidal cells in rat neocortex are extensively coupled via gap junctions. The factors regulating gap junction permeability, as well as the mechanisms underlying the developmental uncoupling process are not understood. To investigate the influence of protein kinase A-mediated phosphorylation on dye coupling in the developing neocortex, pyramidal cells in slices of rat frontal and prefrontal cortex were injected intracellularly with the tracer neurobiotin. Control injections revealed clusters of about 30 dye-coupled neurons. Preincubation with forskolin or direct activation of protein kinase A with Sp-cAMPS reduced the number of coupled cells by about 70%. A significant reduction in dye coupling was also observed following incubation with dopamine. Application of receptor selective agonists and antagonists revealed that the uncoupling was mediated by both dopamine D1 and D2 receptors. The protein kinase A inhibitor Rp-cAMPS reduced the effect of dopamine, suggesting that the neurotransmitter regulates gap junction permeability via protein kinase A activation. In the presence of either forskolin, Sp-cAMPS, or dopamine, neurons displayed a significantly higher input resistance compared to control conditions. During the second postnatal week, transient application of forskolin to single neurons reversibly increased input resistance. At later developmental stages when coupling incidence had declined, this action of forskolin was no longer observed.

Our data demonstrate a dependence of gap junction permeability on protein kinase A activity and on dopamine receptor activation in developing rat neocortical neurons. These mechanisms may modulate junctional permeability during the period of circuit formation.

[Key words: gap junctions, neocortex, development, protein kinase A, dopamine, prefrontal cortex]

Received May 3, 1995; revised July 6, 1995; accepted July 10, 1995.

We thank K. J. Feusner-Treguer for reading the manuscript and G. ten Bruggencate for helpful discussions. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 220/A9).

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The Journal of Neuroscience, November 1995, 15(11): 7386–7400
and reach their targets during the first and second postnatal weeks. These afferents predominantly innervate the superficial cortical layers (Berger et al., 1985). Autoradiographic binding studies have revealed an enhanced expression of dopamine D1 receptors during the second and third postnatal weeks in rat prefrontal cortex (Leslie et al., 1991). A recent in situ hybridization and receptor binding study (Schambra et al., 1994) demonstrated the presence of the PKA-coupled dopamine D1 receptor (D1A) in rat ventricular cells as early as gestational day 14.

The present study was intended to determine whether dye coupling between developing neocortical pyramidal cells is regulated by PKA activation. Following application of either forskolin, membrane-permeable cAMP analogs, or dopamine, we observed a reduction in tracer coupling between superficial pyramidal neurons. The dopamine effect was at least partially mediated via the PKA pathway, suggesting that dopaminergic afferents might be involved in the regulation of electrical and metabolic coupling of neurons during the period of formation of synaptic circuits in the neocortex.

Materials and Methods

Preparation of brain slices. Wistar rats of either sex aged between postnatal days 7 and 15 were deeply anesthetized with diethylether and decapitated. The brain was removed and chilled for 1 min in physiological saline at 4°C. Coronal slices of 500 μm thickness were prepared from the prefrontal and frontoal regions of the cerebral cortex using a vibratome (Campden Instruments, UK). Slices were stored in artificial cerebrospinal fluid (ACSF) at room temperature for at least 1 hr. Individual slices were then transferred to a submerged type recording chamber and placed between two nylon meshes to aid mechanical stability. The slices were continuously perfused with ACSF at a flow rate of 4 ml/min; the chamber volume was 1.5 ml. The ACSF was composed of (in mM): 125 NaCl, 3 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, and 10 glucose. The solution was continuously gassed with carbogen (95% O₂/5% CO₂), resulting in a pH of 7.4 at a recording temperature of 31.5°C.

Neurosipction injections and electrophysiological recordings. To visualize dye coupling between developing neocortical neurons, neurobiotin (Vector, Burlingame, CA) was injected into single neurons. The tracer was ejected iontophoretically from microelectrodes for 10–15 min using subthreshold depolarizing current pulses (100 pA, 300 msec). The microelectrodes had seals with 10% neurobiotin in 1 M KCl. Electrode impedances ranged between 90 and 150 MΩ. The majority of injections was performed in neurons of layers II and III. To investigate the layer specificity of the effect of dopamine, additional injections were made in cells of layers V and VI.

In order to analyze the effects of adenyl cyclase stimulation or direct PKA activation on dye coupling, slices were preincubated for a period of 15–20 min with 20 μM forskolin (Sigma, Deisenhofen, Germany) or 100 μM of the membrane-permeable and phosphodiesterase-resistant cAMP analog Sp-adenosine-3',5'-cyclic monophosphophionate (Sp-cAMPs, Biotrend, Cologne, Germany; Van Hasselt et al., 1984; Wang et al., 1991), respectively. To investigate transmitter effects on tracer coupling, slices were incubated with 100 nM dopamine (Sigma, Deisenhofen, Germany). To prevent oxidation and uptake by endogenous transporters, respectively, dopamine was applied in the presence of both the antioxidant sodium metabisulphite (Na₂S₂O₅, 100 μM) and the uptake inhibitor nomifensine maleate (1 μM, Biotrend, Cologne, Germany). Solutions containing dopamine or receptor selective agonists were renewed every hour. To inhibit PKA activity, the membrane-permeable and phosphodiesterase-resistant cAMP analog Rp-adenosine-3',5'-cyclic monophosphate (Rp-cAMPs, Biotrend, Cologne, Germany; Wang et al., 1991) was applied at least 15–20 min before other drugs were added to the bathing solution.

The D1 receptor-selective agonist SKF 38393 (100 μM, Biotrend, Cologne, Germany) and the D2/3 receptor-specific agonist quinpirole (10 μM, Biotrend, Cologne, Germany) were used to determine the receptor subtypes involved in the action of dopamine. To antagonize dopamine effects, slices were preincubated with either 5 μM of the competitive D1 receptor antagonist SCH 23390 (Biotrend, Cologne, Germany), 10 μM of the D2 receptor antagonist sulpiride (Biotrend, Cologne, Germany), or 30 μM of the combined D1 and D2/3 receptor antagonist haloperidol (Biotrend, Cologne, Germany). The β-adrenergic receptor antagonist propanolol (Biotrend, Cologne, Germany) was used to exclude dopamine effects on β-adrenergic receptors. Slices were incubated in antagonist-containing solutions for at least 15 min before dopamine was added. Neurons were impaled after an additional 15–20 min incubation period. Following neurobiotin injection, the slices were stored for 1 hr in gassed ACSF containing the test substances. Only one neuron was injected in each slice and the duration of tracer injection, as well as survival times after injection were standardized (minimum 10 min injection, 1 hr survival time) to allow for comparison between different slices and preparations. Intracellular recordings were made using a single-electrode current- and voltage-clamp amplifier (NPI SEC-101L, Tamm, Germany). To calculate neuronal input resistance, hyperpolarizing current pulses of 100 pA amplitude and 300 msec duration were injected. Stimulus protocols for intracellular current injection were generated and neuronal responses were digitized using a Digidata 1200 A/D-converter (Axon Instruments, CA) in conjunction with PClAMP software (Axon Instruments, CA). Signals were filtered at 3 kHz and stored on a computer for offline analysis. To investigate drug effects on neuronal properties, the whole-cell blind-patch technique (Blanton et al., 1989; Burgard and Hablitz, 1993) was used. Patch pipettes were pulled from borosilicate glass tubings (Clark Electromedical Instruments, UK) and were filled with a solution containing (in mM): 125 KCl, 0.5 ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 2 MgATP. The pH was adjusted to 7.2. Tip resistances of the electrodes ranged between 4 and 6 MΩ.

Statistical tests were performed using the program INSTAT (GraphPad, San Diego, CA). The significance of differences in electrophysiological properties before and after drug application was determined using the two-tailed Student’s t test. The statistical significance of drug-induced changes in dye coupling was tested using the nonparametric Mann-Whitney U test. Effects of receptor antagonists were analyzed by means of an ordinary ANOVA. Data are given as mean and standard error of mean (SEM). Mean values were considered to be significantly different at p < 0.05.

Histological processing. Following injection and incubation, the slices were fixed for 1 hr in 5% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) and stored over night in 50% sucrose in PBS. To visualize tracer-filled neurons, slices were resectioned at 70 μm on a freeze-cut microtome (2055 Autocut, Leica Instruments). Sections were rinsed (3 x 10 min) in PBS containing NaCl (0.1 M, pH 7.3) and treated (2 x 10 min) with Triton X-100 (0.5% in PBS). The sections were then incubated for 90 min in avidin-conjugated horseradish peroxidase (HRP; Vector, Burlingame, CA; 1% in PBS). Following rinses in PBS (10 min in and in Triton X-100, 0.5 M, pH 8) for 2 x 10 min, the sections were preincubated in 0.5% H₂O₂/0.05% ammoniumbendzine (in Tris/HCl, pH 8) for 15 min. Hydrogen peroxide solution was added at a final concentration of 0.033% The reaction was stopped by rinsing with Tris/HCl (2 x 10 min) and PBS (1 x 10 min). Sections were then mounted on glass slides, dehydrated in ethanol, cleared in xylene, and coverslipped in jung Tissue Freezing Medium (Leica Instruments).

Tissue sections were photographed on a Nikon Biophot (Japan) microscope. A Leitz NPL Fluorat 40x objective and Nikon Plan 20x and 10x objectives were used for photography. Neurobiotin-stained neurons or neuronal clusters were reconstructed using a Leitz Laborlux microscope and a Leitz NPL Fluorat 40x objective with a drawing tube attached to the microscope. Cells showing damage to either soma or principal dendrites were excluded from analysis. Analysis was restricted to cells of comparable staining intensity, i.e., similar diffusion gradients for the tracer can be assumed.

Results

Effects of forskolin and Sp-cAMPs on dye coupling between layer II/III pyramidal neurons

To test whether an increase in intracellular cAMP levels modulates dye coupling, slices were incubated in 20 μM forskolin. Injections were performed in the medial precentral area of the prefrontal cortex, as well as in adjacent frontal areas, and were confined to the superficial layers. Since dye coupling gradually decreases during the developmental period under investigation (Connors et al., 1983; Peinado et al., 1993), data from 2–3 con-
Figure 1. Micrographs of neurobiotin-filled superficial pyramidal cells of the prefrontal cortex injected under control conditions (A) and after incubation in 20 μM forskolin (B and C). The control neuron from a P9 animal showed extensive tracer coupling to neighboring neurons. Dye coupling between pyramidal cells was significantly reduced when injections were performed after 15-20 min incubation in forskolin. Age of animals: B—P9, C—P8. The top of the illustrations represents the pial surface. Scale bar: 50 μm.
Figure 2. Camera lucida reconstructions (A and B) and micrographs (C and D) of a control neuron (A and C) and a pyramidal cell after incubation in Sp-cAMPS (100 µM), respectively. Both cells were located in layer II/III. The reconstructions show the complete morphology and extent of tracer coupling, as well as the distance from the pial surface (dashed line). The control cell (P8) shows widespread tracer coupling (A and C). Incubation with Sp-cAMPS resulted in an almost complete uncoupling of the neuron (P9) shown in B and D. Scale bar: 50 µm.
residual coupling to three other cells is observed. Preincubation in sodium metabisulfite (100 μM) had no significant effect on dye coupling. The neuron (PIO) shown in B displays extensive coupling similar to that observed under control conditions. Scale bar: 70 μm.

Figure 3. Effects of the adenylyl cyclase activator forskolin and the direct protein kinase A activator Sp-cAMPS on dye coupling between lamina II/III pyramidal neurons. Both substances produced a decrease in the average number of coupled cells per injection by approximately 70%. Injections were performed between P7 and P10; the number of cells injected in each group is shown in parentheses. The difference in mean cluster size between controls and pretreated slices was statistically significant (Mann-Whitney U test). In this and the following figures, the bars represent mean ± SEM.

Sequential postnatal days have been pooled. Control injections made on postnatal days 8–10 (P8–10) resulted in a mean number of 30.4 ± 5.1 (n = 14) cells coupled to the injected neuron (range 8–67 cells). After preincubation with forskolin, a marked reduction in cluster size became evident (Fig. 1). On P7–9, the mean number of neurons dye coupled to the core cell was reduced to 7.8 ± 3.3 (n = 19) after forskolin treatment (Fig. 3). The difference between the mean values was statistically significant (p < 0.0001, Mann–Whitney U test). In the presence of forskolin, tracer injection resulted in a single stained neuron in one case. This suggests that the long presence of the transmitter (up to 1 hr) might activate secondary processes leading to prolonged or even permanent uncoupling.

A similar result was obtained when slices were incubated with the direct PKA activator Sp-cAMPS (100 μM). The mean cluster size on P9/10 was 24.1 ± 5.9 (n = 10) under control conditions and 7.7 ± 6.3 (n = 7) in the presence of Sp-cAMPS (Figs. 2 and 3). The mean values differ significantly (p < 0.01). Complete Sp-cAMPS-induced uncoupling was observed in three slices.

Effect of dopamine on dye coupling

To investigate whether dopaminergic mechanisms might be involved in the control of gap junction coupling during cortical development, intracellular injections of neurobiotin were made in the presence of dopamine. The injections were performed in slices obtained from 7–15-d-old animals and were made predominantly in an area of the cerebral cortex receiving strong dopaminergic input, i.e., the medial precentral part of the prefrontal cortex. Figure 4 shows a neuron injected in the presence of nomifensine and sodium metabisulfite (B) as well as neurobiotin-labeled cells after incubation with 100 μM dopamine (A, C). Due to the strong developmental decline in cluster size, neurons were split into three age groups in order to quantify the dopamine effect (Fig. 5). On P8/9, the cluster size was significantly reduced (p < 0.04) from a mean of 37.4 ± 8.0 (n = 7) under control conditions to a mean of 14.0 ± 4.4 (n = 7) in the presence of dopamine. A statistically significant reduction from a mean of 17.6 ± 3.9 (n = 13) coupled neurons to a mean of 5.0 ± 1.3 (n = 8) was also observed on P10/11. Even between P12 and P15, when a strong developmental reduction in cluster size had already occurred, dopamine still produced a statistically significant decline in dye coupling from a mean of 6.4 ± 2.1 (n = 24) coupled cells under control conditions, to 2.0 ± 0.4 (n = 18) in the presence of dopamine. Thus, a dopaminergic modulation of gap junction coupling between layer II/III pyramidal cells is likely to exist during the entire postnatal period.

To investigate reversibility of the uncoupling action of dopamine a sample of P10 neurons was preincubated and injected in the presence of dopamine. Then slices were superfused for a period of 1.5 hr with dopamine-free solution containing only the antioxidant and uptake inhibitor. Under these conditions we have observed only reduced recovery: four out of five neurons were coupled to one to seven neighboring cells, whereas a large cluster containing 26 coupled neurons was observed in only one case. This suggests that the long presence of the transmitter (up to 1 hr) might activate secondary processes leading to prolonged or even permanent uncoupling.

To exclude effects of the added antioxidant and the uptake inhibitor on dye coupling, control injections after incubation in Na2S2O5 (100 μM) and nomifensine (1 μM) without addition of dopamine were performed in slices from rats aged between P7 and P10. These substances had no significant effect on cluster size (Figs. 4 and 5). The mean number of coupled cells under control conditions was 30.4 ± 5.7 (n = 14) and 37.0 ± 9.9 (n = 7) after incubation in antioxidant and uptake inhibitor. Thus, the reduction in cluster size observed after incubation in dopamine can be considered as a specific effect of the neurotransmitter.

Since dopamine applied at high concentrations might exert nonspecific effects via β1-adrenoceptors (Nicol et al., 1990), we injected a sample of neurons in the presence of dopamine...
Figure 5. Effect of dopamine on the size of neurobiotin-labeled cell clusters. Since coupling declines during postnatal development, neurons were split into three age groups. Dopamine induced a statistically significant (Mann-Whitney U test) reduction in the number of coupled cells per injection in all three age groups. Injections performed after incubation of slices in sodium metabisulfite and nomifensine in the absence of dopamine yielded dye-coupled clusters of sizes similar to those observed under control conditions. The number of cells injected in each group is shown in parentheses.

Figure 6. Effects of the protein kinase A inhibitor Rp-cAMPS on dopamine-induced uncoupling of neurons. Injections performed in the presence of dopamine (100 μM) and the protein kinase A inhibitor Rp-cAMPS (100 μM) resulted in dye-coupled clusters (B), which were often of similar size to those observed under control conditions (A). Cells shown in A and B were injected on P8. Scale bar: 50 μm.
intracellular cAMP has been shown to be triggered by a subtype of the D1 receptor, the D1A receptor (Dearry et al., 1990; Monsma et al., 1990; Zhou et al., 1990). We therefore tested the effect of the D1 receptor agonist SKF 38393 on tracer coupling. Preincubation in 100 μM SKF 38393 resulted in a significant reduction in dye coupling both on P7–9 (p < 0.02) and on P11 (p < 0.001, Figs. 8A, C and 9A), suggesting strongly that D1 receptors contribute to the uncoupling action of dopamine. However, the D2/D3 receptor agonist quinpirole (10 μM), although less effective, also caused a statistically significant (p < 0.04) reduction in cluster size (Figs. 8B and 9A) on P7–9.

The effects of dopamine and the selective D1 receptor antagonist SCH 23390 (5 μM), the cluster size was found to be slightly reduced, but not significantly different to controls (Fig. 9B). A weak antagonism of the dopamine effect was also observed upon application of the competitive D2 receptor antagonist sulpiride (10 μM, Fig. 9B). The analysis of variance (nonparametric Kruskal–Wallis ANOVA) revealed that the antagonistic effects of both SCH 23390 and sulpiride were statistically significant (p < 0.03). However, ANOVA posttests (Dunn’s Multiple Comparison Test) showed that the cluster sizes observed in the presence of the antagonists were significantly different from controls, but not significantly different from the cluster size obtained in the presence of dopamine alone. After incubation in dopamine and haloperidol (30 μM), a combined D1/D2 receptor antagonist, the cluster size was found to be not statistically different from control values (p = 0.19) and significantly larger (p < 0.04) than the mean cluster size found in slices incubated exclusively in dopamine (Fig. 9B). Thus, the most effective antagonism of the dopamine effect on dye coupling between superficial layer pyramidal cells in the prefrontal cortex was achieved by blocking both D1 and D2 dopamine receptors.

Since deep cortical layers receive the first dopaminergic afferents during development and the strongest dopaminergic innervation in the adult rat, additional neurobiotin injections were performed in layer V and VI neurons, respectively, to examine layer specificity of the dopamine effect. Control injections in layers V/V1 on P8–10 resulted in a much smaller number of coupled cells per injected neuron compared to superficial superficial layers (2.3 ± 1.32, n = 7, Figs. 10 and 11). Injections performed in the presence of dopamine (100 μM) did not significantly (p = 0.37) change the number of dye-coupled neurons (3.2 ± 0.8, n = 6, Fig. 11).

Effects of dopamine, Sp-cAMPS, and forskolin on membrane potential and input resistance

In order to analyze to what extent dopamine, PKA activating agents, or receptor-selective agonist affect neuronal properties, resting membrane potential and input resistance were measured in control and test solutions prior to intracellular injection of neurobiotin. Figure 13 summarizes data obtained from neurons ranging from P7 to P11. None of the substances tested (dopamine, forskolin, Sp-cAMPS) significantly altered the average resting membrane potential of the neurons. In contrast, the average neuronal input resistance was found to be significantly increased after preincubation in each of these substances (Fig. 13).

Since a reduction in gap junction permeability should change electrotonic cell properties, we investigated the effects of forskolin on electrotonic parameters. Application of 20 μM forskolin reversibly increased neuronal input resistance by 29–165% in five out of nine neurons tested (Fig. 12A). This enhancement in membrane resistance should result in a potentiation of the amplitudes of excitatory postsynaptic potentials (EPSPs). However, since forskolin markedly augments GABA<sub>A</sub>-mediated inhibitory postsynaptic potentials (IPSPs; Penit-Soria et al., 1987; Sutor and Mayr, 1991) and since GABA<sub>A</sub> receptor blockade induces epileptiform activity in the neocortex (Gutnick et al., 1982; Lee and Hablitz, 1991), the effect of forskolin on EPSPs could not be studied directly. We therefore injected transient currents resembling glutamatergic synaptic currents (100 pA amplitude, 1 msec time to peak, 5 msec decay time) and recorded the corresponding voltage responses. These voltage deflections were reversibly potentiated by 33–575% in seven out nine cells (Fig. 12B). Effects of forskolin on current-induced electrotonic potentials were not observed in neurons on P7–11 (n = 6, Fig. 12C, D), i.e., at a time when the extent of dye coupling had declined to a minimum. This finding strongly suggests that the forskolin-induced alterations in electrotonic potentials detected in younger neurons were due to a reduction in gap junctional conductance rather than to an action of forskolin on calcium-dependent potassium currents (Hiramatsu et al., 1994), or voltage-gated sodium conductances (Ono et al., 1995).

Discussion

The present study provides evidence for a regulation of gap junction coupling between superficial pyramidal neurons in rat prefrontal and frontal cortex by protein kinase A. A significant reduction in neuronal dye coupling, as well as a prominent effect on input resistance was observed after adenylyl cyclase activa-
Figure 8. Micrographs of layer II/III cells injected in the presence of the D1 receptor selective agonist SKF 38393 (100 nM; A and C) and the D2/3 receptor selective agonist quinpirole (10 nM; B), respectively. SKF 38393 induced significant uncoupling on both P11 (A) and P7 (C). Quinpirole also significantly reduced dye coupling. The neuron shown in B was injected on P7. Scale bar: 50 μm.

We have used dye coupling as our major assay system to elucidate the mechanisms regulating gap junctional communication. The tracer neurobiotin has been repeatedly used as an indicator of dye coupling between cortical neurons (Peinado et al., 1993). To allow for comparison between individual slices from different animals, we standardized injection and survival times of slices after injection. Only one neuron was injected in...
Figure 9. Effects of dopamine receptor selective agonists and antagonists on dye coupling. A. Effects of receptor selective agonists. The D1 receptor agonist SKF 38393 reduced dye coupling as efficiently as dopamine both between P7 and P9 and on P11. The D2/3 receptor agonist quinpirole was less effective, but the reduction in the number of coupled neurons per injection was also statistically significant (Mann-Whitney U test). B. Effects of receptor selective antagonists. After incubation in dopamine and either the D1 receptor antagonist SCH 23390 or the D2 receptor antagonist sulpiride, the size of neurobiotin-labeled cell clusters was no longer statistically significant from controls (ANOVA, *P* < 0.03). The most effective antagonism of the uncoupling action of dopamine was achieved by incubation in dopamine and the D1/D2 receptor antagonist haloperidol (for *P* values, see text).

Figure 10. Camera lucida reconstruction and micrograph of a layer V neuron injected under control conditions. The camera lucida reconstruction in B demonstrates the distance from the pial surface (dashed line). Already under control conditions dye coupling between deep layer neurons was very weak. Injections were performed on P10, when lamina II/III cells still show extensive coupling. Scale bar: 50 µm.
In order to quantify effects on dye coupling, dopamine was bath applied, and coupling within the entire dendritic tree of the injected cell was analyzed. However, local uncoupling due to transmitter release at single modulatory terminals affecting only single dendrites or even parts of dendrites is likely to occur under physiological conditions. Although dopamine is released predominantly from varicosities and might reach its receptors by diffusion over relatively large distances, dopaminergic terminals have also been shown to be involved in triadic synaptic complexes in both rodent (Verney et al., 1990) as well as primate (Goldman-Rakic et al., 1989) prefrontal cortex, suggesting highly localized effects of the transmitter. The distance between dopaminergic release sites and gap junctions, however, is not known to date, and whether dopamine receptors are located in close proximity to junctional complexes also remains to be shown. Since dopamine receptor-mediated uncoupling of gap junctions might occur via widespread “volume neurotransmission” (i.e., undirected spread of transmitter in a larger volume of cortical tissue; Lidow, 1995) or in a highly localized fashion, it is unclear whether activation of dopaminergic afferents uncouples an assembly of neurons, e.g., an entire column, single neurons, or only local dendritic compartments from the functional syncytium.

We have only analyzed the effects of exogenous transmitter on dye coupling without attempting to stimulate release from dopaminergic terminals in the slice preparation. Dopaminergic fibers originating in the brainstem are cut during preparation and the diffusely projecting dopaminergic afferents are difficult to stimulate. The dopamine uptake inhibitor nomifensine did not reduce dye coupling, indicating that spontaneously released dopamine does not reach concentrations high enough to significantly affect gap junction coupling. Microelectrodes rather than patch pipettes were used for tracer loading of pyramidal cells to exclude false negative results due to washout of components of intracellular second messenger pathways. However, to obtain higher resolution recordings, we used the patch-clamp technique to study the influence of gap junction closures on electrotonic potentials. Under these conditions, forskolin, which most effectively reduced tracer coupling, exerted effects on electrotonic potentials in five out of nine neurons tested. The lack of effect in four cells might be due partially to a long electrotonic distance between the location of gap junctions and the recording site, although a dialysis of intracellular components might also have taken place by the time PKA-mediated phosphorylation reduces junctional communication.

Cyclic adenosine monophosphate and dopamine as negative regulators of gap junctional communication during postnatal development of the neocortex

In different tissues, PKA stimulation has heterogeneous effects on gap junction coupling. Whereas coupling is enhanced in some non-neuronal tissues (DeMello 1983; Saez et al., 1986; Burt and Spray, 1988; DeMello 1989), an inhibitory effect of PKA activation has been shown in the vertebrate retina (Ieramshii et al., 1983; Piccolino et al. 1984; Lasater and Dowling, 1985; Lasater, 1987; DeVries and Schwartz, 1992; Hampson et al., 1992; McMahon, 1994). Our results demonstrate a suppressive effect of raising intracellular cAMP concentrations on dye coupling in the developing rat neocortex. This second-messenger pathway is regulated by a number of G-protein–coupled neurotransmitter receptors, suggesting that chemical and electrical communication systems are closely interrelated in the immature neocortex.

Figure 11. Dye coupling between deep layer pyramidal neurons. The number of coupled neurons per injection was significantly lower in comparison to superficial neurons. Dopamine had no statistically significant effect on tracer coupling in layer V/VI neurons (Mann-Whitney U test).

Each slice and only pyramidal-shaped neurons were included in the analysis. The extent of coupling was assessed by counting the coupled somata in the vicinity of the injected neuron.

To investigate transmitter effects on dye coupling, slices were incubated for 1.5 to 2 hr, including preincubation and survival times, in ACSF containing a rather high agonist concentration. Therefore, desensitization of receptors resulting in an underestimation of the effect cannot be excluded. However, the reduction in dye coupling was of high statistical significance. We used high agonist concentrations mainly for two reasons: (1) dye coupling displays high variability already under control conditions, and (2) receptor densities might be low as compared to the adult cortex. Furthermore, among the various dopamine receptor subtypes, the affinity for dopamine is lowest at the D1 and D2 receptors (Schwartz et al., 1992). The dopamine-induced uncoupling could be mimicked by both the D1 receptor selective agonist SKF 38393 and the D2 receptor selective agonist quinpirole. It was also observed in the presence of the β-adrenoceptor antagonist propranolol. These findings provide strong evidence for a dopamine effect selectively mediated by dopamine receptors. Since we used high agonist concentrations, the observed uncoupling effect of dopamine could not be completely suppressed by receptor selective antagonists applied in the “normal” pharmacological concentration range. Nevertheless, in neurons pretreated with dopaminergic antagonists, the reduction in cluster size induced by subsequent treatment with dopamine failed to reach statistical significance. In retinal amacrine cells, the antagonizing effect of SCH 23390 has been shown to depend strongly on the dopamine/SCH 23390 concentration ratio (Hampson et al., 1992). However, we did not further increase antagonist concentrations, since this might have resulted in a loss of receptor subtype selectivity.
Figure 12. Effects of forskolin application on electrotonic potentials. On P7–11, forskolin induced a marked and reversible increase in input resistance in five out of nine cells tested (A). Voltage responses to transient depolarizing currents were also reversibly potentiated (B). Effects of forskolin on electrotonic potentials were no longer observed in neurons on P17/18 (C and D), suggesting that the changes in electrotonic parameters observed in younger neurons were due to gap junction closure. Recordings were performed at resting membrane potential (−65 mV in A and B, −73 mV in C and D).
Thus, the most likely explanation for the reduction in dye coupling induced by PKA in neocortical neurons is phosphorylation of connexins, which are endowed with several serine phosphorylation sites (see Bayer, 1993, for review).

The uncoupling effect of dopamine in the developing rat neocortex was mediated by both dopamine D1 and D2 receptors. D2 receptors are known to reduce intracellular cAMP concentrations (Onali et al., 1984; Battaglia et al., 1985; Weiss et al., 1985); however, a linkage to other second-messenger pathways has been shown (Piomelli et al., 1987; Kanterman et al., 1991; Jackson and Westlind-Danielsson, 1994). The application of receptor selective agonists demonstrated that activation of either D1 or D2 receptors is sufficient to depress dye coupling. Therefore, an intracellular signal transduction system different from the cAMP pathway might be involved in the action of dopamine on gap junction permeability. Possible candidates are protein kinase C-dependent phosphorylation, calcium ions, or arachidonic acid metabolites (Rose and Loewenstein, 1975; Rao et al., 1987; Miyachi et al., 1994).

**Figure 13.** Comparison of mean membrane potentials (A, ± SD) and input resistances (B) of neurons (P7-11) under control conditions and after preincubation with forskolin, Sp-cAMPS and dopamine. Whereas resting membrane potentials were not significantly altered, all three substances produced a statistically significant increase in input resistance (two-tailed Student's t test) in lamina II/III neurons as compared to age-matched control neurons. In neurons of deep cortical layers, dopamine did not significantly increase input resistance. Statistical significance is indicated by asterisks.

We have not directly demonstrated a PKA-mediated phosphorylation of connexins, i.e., the observed reduction in dye coupling might be indirectly induced via phosphorylation of a different protein. Stimulation of the PKA pathway via D1 receptors has recently been shown to modulate voltage-activated calcium currents in striatal neurons (Surmeier et al., 1995). However, depolarizing pulses applied to inject neurobiotin were below spike threshold, thus preventing calcium entry during the action potential. Since the dopamine-induced reduction in dye coupling was antagonized by the PKA inhibitor Rp-cAMPS, an indirect effect via changes in intracellular or extracellular pH can be excluded. It has been shown that activation of adenylyl cyclase and PKA results in a decrease in open probability of gap junction channels in retinal neurons (Lasater, 1987; McMahon and Brown, 1994). Thus, the most likely explanation for the reduction in dye coupling induced by PKA in neocortical neurons is phosphorylation of connexins, which are endowed with several serine phosphorylation sites (see Bayer, 1993, for review).

During the early postnatal period, dopaminergic terminals are present in both superficial and deep cortical layers (Berger et al., 1991). The strongest expression of both D1 and D2 receptors occurs in the deep layers, although these receptors are also present in the superficial layers of prefrontal areas (Schambra et al., 1994). A significant increase in D1 receptor expression has been observed between the second and third postnatal week when gap junction coupling disappears and synaptogenesis increases (Leslie et al., 1991). Thus, the dopaminergic fiber system might exert a twofold function: at embryonic and early postnatal stages, dopamine might regulate metabolic and electrical signal transfer, and, by regulating electrotonic cell parameters, it might affect the efficacy of developing chemical synapses.

Layer V/V1 pyramidal cells were already uncoupled, and dopamine had no significant effect on residual tracer coupling in the deep layers during the second postnatal week, suggesting that uncoupling follows a developmental gradient reflecting the “inside-first outside-last” pattern of neurogenesis in the neocortex.

Dopamine has been suggested to act as a neurotrophic factor during development of the neocortex, since lesioning of dopaminergic afferents reduces cortical thickness (Kalsbeek et al., 1987) and impairs the development of cortical neurons (Kalsbeek et al., 1989). In the adult rat prefrontal cortex, dopamine potentiates GABAergic synaptic transmission via a presynaptic mechanism (Penit-Soria et al., 1987; Sutor and Mayr, 1991) and shifts the balance between long-term potentiation and long-term depression in favor of long-term depression (Law-Tho et al., 1995). Thus, the functions of the dopaminergic projection to the neocortex obviously change during development.

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