Laminar and Temporal Heterogeneity of NMDA/Metabotropic Glutamate Receptor Binding in Posterior Cingulate Cortex

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1Department of Neurology, 2Department of Neuroscience, and 3Department of Pediatrics, Albert Einstein College of Medicine; and 4Center for Epilepsy Management, Montefiore Medical Center, Bronx 10461; 5Mercy College, Dobbs Ferry, New York 10522; and 6Novartis Pharmaceuticals, East Hanover, New Jersey 06851

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Hedberg, Thomas G., Ellen F. Sperber, Jana Velůšková, and Solomon L. Moshe. Laminar and temporal heterogeneity of NMDA/metabotropic glutamate receptor binding in posterior cingulate cortex. J Neurophysiol 84: 1881–1887, 2000. Both N-methyl-D-aspartate (NMDA) and quisqualate/AMPA-insensitive metabotropic glutamate (mGlu) receptors mediate plasticity induction in neocortex, but their interlaminar distribution in cortical microcircuits is largely unknown. We used (+)-3H-MK801 and 3H-glutamate binding plus saturating concentrations of NMDA, AMPA, and quisqualate to autoradiographically map NMDA and mGlu receptor sites by lamina in posterior cingulate cortex in adult male rats. Specific binding at NMDA receptor sites in laminae II/III and VI was significantly reduced in comparison to other laminae. Brains prepared from rats killed during dark phase of a 12h/12h light/dark cycle showed a mean 129% increase versus light phase binding but did not retain significant contrasts to NMDA findings, specific binding at mGlu sites was consistently elevated during light phase in both laminae II/III and VI. Specific 3H-glutamate binding in dark-phase brains showed an overall 147% increase versus light phase binding but did not retain significant interlaminar heterogeneity. Interpreted in accordance with our physiologically derived models of hippocampo-cortical microcircuitry, these results suggest that spatial and temporal variations in glutamate receptor distribution may play an important role in intracircuit neural processing of afferent input from hippocampus.

INTRODUCTION

Posterior cingulate cortex (PCC) or cortical area 29 (Brommann 1909) is involved in associative learning and memory (Gabriel et al. 1987), affect (White and Sweet 1969), and pain mechanisms (Khachaturian et al. 1983). Prominent afferents to PCC include excitatory projections from subiculum to lamina II/III via the subiculo-cingulate tract (SCT), a nonreciprocal affenter projection, and from anterior thalamic nuclei to laminae I and V (Peters and Jones 1985). Numerous synaptic mechanisms in PCC underlie the processing of inputs driven by SCT excitation. Intracellular and single-unit recordings show that single-pulse subicular stimulation elicits markedly asynchronous responses in superficial (II/III-IV) and deep (V-VI) laminae pyramidal neurons (Hedberg and Stanton 1995). Although SCT terminates almost exclusively in superficial lamina II/III, pyramidal cells with somata and basal dendritic arborizations in this region do not respond monosynaptically to subicular stimulation but show polysynaptic mixed excitatory/inhibitory postsynaptic potentials. In contrast, deep laminae neurons with superficially ramifying apical dendritic arbors show mono- and disynaptic excitatory drive (Finch et al. 1984; Hedberg et al. 1993). These findings suggest that PCC may process excitatory input from SCT via a synaptic loop. Mono- and disynaptic excitation of deep lamina pyramidals neurons by their apical dendritic arbors allows transmission, via ascending axonal collaterals, of largely unmodified hippocampal input to neural plexi in superficial laminae for processing and re-excitation. In vivo as well as in vitro tests of predicted PCC microcircuit response properties have supported these interpretations (Finch et al. 1984; Hedberg and Stanton 1995; Hedberg et al. 1993).

In addition to acute modulation of input signal processing, excitatory amino acid neurotransmitters are responsible for induction, expression and maintenance of synaptic plasticity. N-methyl-D-aspartate (NMDA)-type ionotropic receptors mediate most long-term potentiation (LTP) in mammalian CNS (Harris et al. 1984; Monaghan et al. 1989). Metabotropic glutamate receptors have been implicated in the induction of LTP as well as long-term depression (LTD) (Kato 1993; Zheng and Gallagher 1992). Experiments using the mGlu blocker DL-AP3, and the NMDA blocker D-AP5 indicate that while monosynaptic responses to SCT stimulation require both mGlu and NMDA receptors for plasticity induction, a potentially distinct population of di- or trisynaptically activated deep lamina pyramidals neurons (not driven directly by SCT input), may require only NMDA receptors. These synapses do not lose their plasticity when mGlu sites are blocked (Hedberg and Stanton 1996). Despite intriguing suggestions provided by these electrophysiologic findings, the interlaminar distribution and expression of these receptors has not yet been determined in PCC.

Receptor expression can show marked circadian variability (Holmes et al. 1997; Masson-Pevet et al. 1996), and glutamate receptors should be mapped temporally, as well as spatially, onto microcircuit models of PCC. Early work by Kafka et al. (1983, 1986) has demonstrated that receptor Bmax can display a strong diurnal oscillation in the plasma membrane. In PCC, these oscillations in receptor expression are likely to reflect interlaminar receptor distribution and function.
muscimol and baclofen binding sampled at two midpoints of a light/dark cycle showed that GABA receptors followed a daily shift in binding affinity (Hedberg and Vogt 1988). Glutamate receptors in this region may show similar patterns of circadian variability.

Assembling our earlier work on the involvement of PCC in signal processing and gating, the role of glutamate receptors in plasticity induction, and the influences potentially exerted on both functions by diurnal oscillations in receptor availability, the fine spatial and temporal mapping of glutamate receptors in PCC is a critical step toward refinement and verification of models of hippocampal-cortical interaction. The present work addresses four outstanding issues: 1) since neuronal processing in PCC is highly stratified by lamina, we expect receptor distribution to be correspondingly heterogeneous and segregated both by origin and terminal field of major projections; 2) distribution and density of NMDA versus mGlu receptors should correspond with circuitry mediating induction of long- and short-term plasticity induction in PCC; 3) we expect light-phase/dark-phase differences in binding to reflect diurnal oscillations in receptor expression/affinity and affects on information processing; 4) glutamate receptor distribution should complement processing, potentiation, and gating functions in PCC and shed light on mechanisms governing conductance generalization of epileptic seizures having a complete or partial origin in hippocampus.

In the present study we used quantitative tritium autoradiography (grain counts) to provide an accurate and quantifiable picture of receptor distribution and density across neocortical laminae in adult rats kept on a light/dark diurnal cycle. The fine spatial resolution achieved with this technique is necessary for interlaminar discrimination of receptor binding and responsiveness to potentially small circadian variabilities in binding pattern and density.

METHODS

Tissue preparation

Adult male Sprague-Dawley rats (150–250 g) were maintained for 10–14 days prior to death on a 12h light/12h dark cycle. To prepare tissue for binding, half the animals were decapitated in the middle of their subjective day (photoperiod). The remaining animals were killed at the midpoint of their subjective night (scotoperiod). Brains were removed, and sections containing PCC were frozen in methylbutane at −35°C and stored at −70°C.

Binding assays

Frozen blocks were cut at −20°C. Coronal sections containing most of the rostrocaudal extent of PCC were cut 20 μm thick on a cryostat and then thaw-mounted on chrom-alum subbed glass slides, three sections to a slide. Mounted sections were used for binding after warming to room temperature. To initiate binding assays, all slides were first preincubated in 50 mM Tris-HCL (pH 7.4), 2.5 mM CaCl2, and 30 mM KSCN (or 100 mM thiocyanate), for 40 min at 4°C to enable dissociation of bound, endogenous ligand (Hedberg and Vogt 1988). Sections were then dried with a stream of warm air.

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inspection of the more distal aspects of the section, and a seventh and final count was taken from a centrally placed window.

Counts due to artifact and overlapping grains were both visually corrected and filtered out of the count by presetting the grain size window. Uniformity of emulsion was determined for each section by subtracting mean grain counts made on four regions of the coverslip, two above and two below each tissue section under study. Specific binding was determined by further subtracting nonspecific from total binding for each area of interest. Mean specific binding was calculated for each region studied, and a mean ± SE was calculated for each group of animals. Interlaminar variations in binding were assessed by normalizing each section to a 100% baseline and then comparing laminar binding on the basis of percent change (%Δ) from baseline for mean values compiled for each lamina. Further analysis involved a second internal normalization in which z-score values (Hay 1963) were computed on the basis of consistent variation from a hypothetical trans-laminar null value. Interlaminar heterogeneity was assessed for significance using Student’s t-test. Slide-coverslip packages were photographed using both transillumination (brightfield) and side-illumination (darkfield). Kᵣ and Bₘₐₓ values were plotted by Scatchard analysis.

RESULTS

NMDA receptor binding

Coronal sections from 10 animals were used: 7 taken at mid-photoperiod, and 3 taken at mid-scotoperiod. Each brain provided ~40 sections. In sections exposed to (+)³H-MK801 (plus cold-chase ligand for specific binding), patterns of grain density reflecting receptor distribution were evident under darkfield visual inspection. Binding at VM sites was dense both in anterior (bregma: 2.5–5.4) and posterior (bregma: 5.5–7.7) sections of PCC. In contrast, control binding was low (<10% of specific) in all sections. There was no evidence of significant interlaminar variations in control binding in anterior or posterior sections taken from animals killed during the midpoints of either photoperiod or scotoperiod.

Specific binding was consistently pronounced in molecular sublaminae Ia through Ic (a largely acellular region of dendritic arborization), where quantification of each scanned section gave a mean of ~4,000 grains/480 μm². Since per-lamina grain counts were a function of overall binding density (which did vary between slides with emulsion thickness and radioisotope penetration variables), data for each section were normalized to specific binding in lamina Ia–c. Mean specific binding per lamina could thus be calculated as a percent change from the lamina Ia–c reference baseline for each section. Visual examination of interlaminar grain distribution and density in 6–12 separate sections indicated a uniform diminution in laminae II/III and VI binding density across the rostrocaudal extent of PCC (Figs. 1 and 2). Per-lamina assessments of specific binding for each condition confirmed these observations.

As shown in Fig. 2A, normalization of binding data revealed a notable decrease, versus lamina Ia–c reference baseline, in binding density in lamina II/III (Δ%Δ = −10.2 ± 0.6%; mean ± SE; P < 0.02) in sections taken from photoperiod animals. Likewise, binding in lamina VI showed a concomitant drop versus baseline of Δ%Δ = −17.7 ± 3.5% (P < 0.05). A secondary internal normalization within each section was performed to obtain a z score for mean binding in each lamina. Again, significant comparative decrements in binding in laminae II/III and VI indicated that (+)³H-MK-801 binding was consistently limited in both laminae. In other laminae, grain density varied both higher and lower than the Ia–c baseline and revealed no consistent increases or decrements in laminar-specific binding.

At mid-scotoperiod, an overall increase in specific (+)³H-MK801 binding was seen, with binding density ~5,000 grains/480 μm² tissue area. Mean mid-scotoperiod binding averaged 128% of mean mid-photoperiod binding, indicating that a substantial change in receptor binding affinity or receptor availability may occur across a circadian time frame. As seen in Fig. 2B, normalized binding densities recorded in mid-scotoperiod sections retained the same interlaminar proportions seen in mid-photoperiod sections and continued to show a pronounced and significant drop in lamina II/III (Δ%Δ = −10.1 ± 2.3%; P < 0.02) and VI (Δ%Δ = −4.8 ± 1.3%; P < 0.05; Fig. 2B).

mGlu receptor binding

In contrast to binding density patterns observed using (+)³H-MK801, specific binding of ³H-glutamate was markedly reduced overall. In both photoperiod and scotoperiod brains, specific ³H-glutamate binding was three to six times less than the density of specific (+)³H-MK801 binding although all conditions showed approximately the same degree
of nonspecific binding. Visual assessment of 3H-glutamate-bound sections showed grain distribution to be rather diffuse. Notwithstanding, quantitative analysis revealed a consistent pattern of specific binding different from that seen in NMDA sections.

Using specific binding in lamina Ia–c as baseline, sections obtained in mid-photoperiod showed consistent increases in 3H-glutamate binding density in laminae II/III through VI (Fig. 3A). Normalized binding is calculated as a percentage change from lamina Ia–c baseline grain count for each section scanned (n = 8). Note: SEs of ≥5.0% and less do not appear on bar graph.

FIG. 3. Normalized interlaminar MGLU binding in PCC: mean specific normalized interlaminar binding of 3H-glutamate in adult rats killed at mid-photoperiod (A) and mid-scotoperiod (B). □, nonspecific binding; *, statistically significant changes from baseline. Normalized binding is calculated as a percentage change from lamina Ia–c baseline grain count for each section scanned (n = 7). Note: standard errors of ≥5.0% and less do not appear on bar graph.

Grain-density patterns quantified in this study appear to represent physiologic heterogeneities in the binding of glutamate radioligands to specific receptor sites for the following reasons: nonspecific binding was consistently low and largely invariant among animals, sections, and slides for both NMDA and mGluR sites; laminar-specific heterogeneities in binding density were similar from section to section across all animals; and mGlu binding showed increases at the same locations where NMDA binding showed decreases. Together these factors imply a physiologic etiology rather than tissue damage or artifact and suggest that radioligand binding occurred at specific receptor sites.

Interlaminar heterogeneities in binding density may reflect circadian oscillations in both pre- and postsynaptic receptors that impose cyclic short-term changes in the functional integrity of PCC microcircuits. Diurnal oscillations in neurotransmission of nonspecific binding sites.
miter expression, genetic composition, binding affinity, and mechanism of action have been reported in hippocampus and elsewhere for GABA<sub>A</sub> (Kanterewicz et al. 1995); 5-HT<sub>2c</sub> (Holmes et al. 1997), melatonin (Masson-Pevet et al. 1996), and NMDA (Ding et al. 1998) receptors. Oscillations in receptor binding and action have been linked with mood and affective states (Borsook et al. 1984) and are critical to the linking of photic and other time cues with endogenous neurohormonal oscillations (Wirz-Justice 1987). The nocturnal up-regulation of receptor availability/affinity seen in this study during the animal’s subjective night, yielded an oscillating ratio of NMDA (primarily LTP) to mGlu (primarily LTD) receptor binding. This, along with concomitant loss of the interlaminar 3H-glutamate binding heterogeneity characteristic of photoperiod binding, could significantly influence the expression of long-term plasticity in PCC information-processing networks.

The anatomic findings discussed in the preceding text may acquire greater significance in light of earlier in vitro (Hedberg et al. 1993) and in vivo (Hedberg and Stanton 1996) electrophysiologic work. In both studies cited, the response of PCC to subicular stimulation was stereotypic and highly influenced by the frequency/amplitude of synaptic inflow via SCT. When stimulation frequency exceeded 8 Hz (or dropped < 4 Hz), processing subroutines were elicited in superficial laminae, and the discharge rate of polysynaptically driven deep laminar (V-VI) pyramidal cells became asynchronous. However, when stimulation frequency moved into the 4- to 8-Hz window (approximating the theta spikes seen in EEG records), both superficial and deep laminar neuronal discharge became coincident. As shown in Fig. 5, this direct relay of a narrowband of amplitude and frequency-modulated signals enabled monosynaptically driven pyramidal output to extracortical targets to become essentially synchronous, of enhanced amplitude, and liable to LTP induction.

The existence of a mechanism which enables plasticity induction in deep-laminar pyramids is supported by the sharp reduction in NMDA receptor representation in the principal terminal field of SCT (lamina II/III). Since NMDA-type glutamate receptors are the primary transducers of LTP induction in neocortex (Dudek and Bear 1992), it is reasonable to posit that their representation should be reduced where the microcircuitry of PCC is first activated. Otherwise plasticity at this

**FIG. 4.** NMDA binding in 29c/MGLU binding in 29c: comparison of NMDA and mGlur binding across all 6 experimental populations (n = 6–8) for all conditions. Figure depicts means of total specific binding in each lamina prior to normalization and shows consistency of drop in binding density between adjacent laminae Ia–c and II/III in all experimental conditions.
primary synaptic junction could alter input to PCC and compromise its information-processing function. The relative increase in co-localized mGlu receptor sites becomes important in this context. Since mGlu receptors are known to mediate LTD (Kato 1993), a nullifying balance between the two receptors, augmented by the inhibitory influence of local-circuit interneurons, could account for nonplastic monosynaptic activation of PCC by frequency/amplitude coded hippocampal input.

This same model may explain the dense binding at NMDA sites in laminae I and Vb. If monosynaptic excitation by SCT of lamina V pyramidal neurons is nonplastic at apical dendritic synapses (Hedberg and Stanton 1996), polysynaptic activation must be responsible for long-term changes. Thus as modeled in Fig. 6, SCT-driven LTP/LTD could arise in deep lamina pyramidal neurons if: the synaptic input required to induce plasticity was not disrupted by upper laminar PCC processing (e.g., during theta-frequency tetanic stimulation or “theta-gating”) and plasticity-inducing SCT input was relayed to deep lamina pyramidal neurons by NMDA receptors at dendritic/somatal synapses formed by axon collaterals of monosynaptically driven lamina Vb neurons. In support of this, the field potential representing SCT-elicited discharge of deep-lamina PCC pyramidal neurons is best potentiated by theta-pulse tetanic stimulation (Stanton et al. 1994); inhibited from LTP by the NMDA-specific antagonist D-AP5; and unaffected by the mGlu-specific antagonist DL-AP3 (Hedberg and Stanton 1996). Large pyramidal neurons in lamina VI comprise the final extracingulate output for PCC-processed information (Donovan and Wyss 1983; Meibach and Seigel 1977). Synaptic inflow converging on their basal dendrites arises largely from extracingulate sources (e.g., anterior thalamic nuclei) while intracingulate projections terminate at their highly branched and extremely spiny dendritic tuft arborizations in lamina Ia-c.

Responses to PCC processing would occur primarily at subpial apical tufts, while basal dendritic (extracingulate) inputs should be plasticity-free.

Disruption of the integrity of this system may contribute to the etiology of certain convulsive disorders. An interesting question bears on how (given the glutamate receptor distribution mapped in this study) models of plasticity induction and theta-gating in PCC can account for the amplification or inhibition of propagated rhythmic/epileptiform neural activity. In healthy (nonepileptic) brains, gating may be abnormal (thalamic quiescence and/or facilitation of unmodified 1:1 SCT throughput), and damping may be lost, a potentially catastrophic circumstance given PCC’s multiple projections to thalamus, motor cortex, pons, periaqueductal gray, etc. (Swanson and Cowan 1977).

The synchronous and rhythmic discharge of large populations of neurons during an epileptic seizure may be akin to our 4- to 8-Hz stimulation protocol. While theta frequency input itself cannot give rise to synaptic plasticity, Stanton et al. (1994) have shown that “theta-burst” stimulation most effectively induces hippocampal LTP. Theta-burst stimulation uses a frequency-modulated 4- to 8-Hz signal containing an amplitude-modulated component (the discharge frequency of neurons represented within each successive wave of afferent excitation). With a failure of superficial modulatory mechanisms, not only can theta frequencies open up a 1:1 throughput conduit for epileptic output from hippocampus, but the presence of 80- to 120-Hz excitatory

FIG. 5. Schematic representation of AM/FM signals in theta gating activity within posterior cingulate cortex. “Input” represents action potentials carried by SCT afferent inflow from subiculum to apical dendritic arbors of deep laminae pyramidal neurons. “Output” represents action potentials carried by extracingulate afferent axonal projections originating in deep laminar (Vb, VI) pyramidal neurons. Frequency of tetanic signals yielding potentiated or complex responses in this model are approximate.

FIG. 6. Schematic representation of NMDA and mGlu glutamate receptor distribution in PCC. Forks, synaptic terminals; triangles, pyramidal neurons associated dendrites and axon collaterals; black circles, inhibitory interneurons and associated local circuit axons and dendrites. Bold font indicates regions of dense binding; normal font indicates regions of relatively sparser binding. Note that NMDA density is highest in lamina Ia–c and lamina V-VI, while mGlu density is highest in lamina II/III. Potentiated or complex responses in this model are approximate.
activity within each theta wave can potentiate that pathway, making it more susceptible to future epileptic activity. Potentiated, theta-gated activity, strengthened and facilitated by its passage through PCC, may contribute significantly to seizure generalization.

These findings may have intriguing long-range applications. If theta-gating dysfunction has a pharmacologic base (such as NMDA, mGlu or AMPA hypo/hyperexpression), drug-based treatments are indicated. Given a laminar-specific circadian oscillation in binding patterns, maximal efficacy of any such pharmacotherapy might be achieved by appropriate timing of drug administration. Seizure activity is often linked to a specific time of day, and such oscillations in facilitatory pathways may play a role in these temporal characteristics. Should the etiology encompass dysfunction in the microcircuit processing of AM/FM characteristics of thalamic/hippocampal inputs, an electrophysiologic route is plausible; e.g., an implantable, frequency-sensitive neuroprosthesis could be tuned to respond to preictal signals by emitting a burst of de-synchronizing stimuli in critical neocortical regions. Additional clarification of the cellular distribution of plasticity-sensitive glutamate receptors within the PCC microcircuit will permit a much more profound understanding of the way this critical hippocampal-cortical interface functions.

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