Neurochemical Research, Vol. 21, No. 2, 1996, pp. 285–291

High Levels of Extracellular Glutamate Are Present in Retina During Neonatal Development*

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(Accepted December 12, 1995)

The three major classes of neurons which comprise the primary visual pathway in retina are glutamatergic. These cells are generated in two separate developmental stages, with one subclass of photoreceptors (cones) and ganglion cells generated before birth; and the other subclass of photoreceptors (rods) and bipolar cells generated during the first week after birth. Gas chromatography/mass spectroscopy analysis coupled with a new method for collecting small samples of extracellular fluids from retina were used to determine the levels of endogenous glutamate present during differentiation and synaptogenesis of these different cell types. As expected the total retinal content of glutamate increased during the postnatal period in synchrony with the generation and maturation of glutamatergic cells. However, a significant proportion of the endogenous pool was found extracellularly at birth. Intracellular glutamate is localized within cell bodies and growing processes of cones and ganglion cells at this time but few glutamatergic synapses are present. The extracellular concentration of glutamate actually declined during the most active period of synaptogenesis, reaching very low levels in the adult. The high concentrations of extracellular glutamate in neonatal retina could play an important role in a variety of developmental events such as dendritic pruning, programmed cell death and neurite sprouting.

KEY WORDS: Glutamate; retinal development; glutamate release; retina.

INTRODUCTION

The amino acid neurotransmitter, glutamate, functions as a major excitatory synaptic signal in the retina (1). The retina is a highly useful model for characterizing neurotransmitter systems in the CNS because of its simple organization, its well-characterized circuitry, and its suitability for experimental manipulation. In the retina, glutamate mediates synaptic transmission from photoreceptors to bipolar cells and from bipolars to ganglion cells as part of the primary visual pathway. The action of glutamate is mediated via one of several subtypes of postsynaptic receptor (for review, see 2).

In addition to conventional synaptic transmission, a spectrum of activity has been demonstrated for glutamate depending on the concentration of transmitter and the maturational stage of the tissue. High concentrations of glutamate produced in response to ischemia, activate the NMDA subclass of glutamate receptors and mediate glutamate neurotoxicity in both the adult retina and brain (3,4,5). The NMDA receptor may also participate in specific developmental processes in brain ranging from dendritic pruning to neurite extension (6). By using receptor antagonists, the NMDA receptor has been shown to modulate the organization of the retinotectal map (7,8) and the refinement of the synaptic connections in the striate cortex (9).

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* Special issue dedicated to Dr. Kinya Kuriyama.

0364-3190/96/0200-0285$09.50/0 © 1996 Plenum Publishing Corporation

In early postnatal development of the retina, various concentrations of glutamate agonists appear to mediate a range of activities. Both NMDA and non-NMDA receptor activation alters electrical activity and intracellular calcium levels of ganglion cells (10). Messersmith and Redburn (11) demonstrated that at high concentrations the non-NMDA agonist, kainic acid, ablated rabbit retinal horizontal cells early in development. In the rat hippocampus (12) and retina (13) low concentrations of kainic acid act as a trophic agent and in retina, it induces neurite sprouting of horizontal cells.

The presence of glutamate immunoreactivity has been demonstrated in the developing rabbit retina by Redburn et al. (14). Labeling was observed in the cell bodies and pedicles of cone photoreceptors, the cell bodies and processes of horizontal cells and in ganglion cells which together constitute the first cohort of neurons generated embryonically. Many of the cell types produced postnatally in the second wave of neurogenesis (i.e. all rod photoreceptors and bipolar cells as well as many amacrine cells) are also immunoreactive for glutamate within the early postnatal period. In horizontal cells and some amacrine cells, glutamate may serve as a precursor for the production of GABA rather than acting directly as a neurotransmitter (15).

In the present study, the primary objective was to measure the size of the total pool of endogenous glutamate and the fraction of that pool found extracellularly during development in the rabbit retina. In order to assay extracellular glutamate, we adapted a technique first developed for brain slices (16). We report that the extracellular pools are relatively large during early post natal development and decrease in size as the neuronal and glial architecture matures. Furthermore, the diminishing pools of extracellular glutamate constitute only a portion of the total glutamate content which in fact increases during development. The relatively high concentration of glutamate found extracellularly during development further supports a role for glutamate in retinal and CNS maturation.

EXPERIMENTAL PROCEDURE

Superfusion of Isolated Retina. New Zealand white rabbits of varying ages were obtained from a local vendor and handled in accordance with the guidelines set by the Association for Research in Vision and Ophthalmology. Animals were anesthetized with CO_2, decapitated, and immediately pithed by mechanical disruption of brain stem structures with a blunt probe inserted through the foramen magnum. The eyes were removed and hemisected posterior to the ora serrata. The anterior portion of the eye and vitreous were discarded. The posterior eye cup was flattened by making four radial cuts. Four-ply gauze was placed over the vitreal surface. The sclera was then carefully peeled back with a pair of forceps leaving the retina positioned photoreceptor side up on the gauze. The gauze and retina were placed over a clear plastic dome leaving the outer surface of the retina exposed to moist air inside an incubation chamber maintained at 37°C (Fig. 1). The level of the buffer in the chamber was controlled by inlet and outlet valves, so that it reached only the edge of the gauze. The wicking action of the gauze served to keep the retina supplied with oxygenated buffer (95%, O_2, 5% CO_2). In all experiments, the buffer used was Krebs bicarbonate (pH 7.4), which contained 4.7 mM KCl, 1.17 mM KH_2PO_4, 118 mM NaCl, 11.1 mM glucose, 1.2 mM $MgSO_4$, and 2.5 mM $CaCl_2$. A micropipette was positioned along the outer retinal surface and 10 µl samples of surface fluid were collected manually. The same pipette arrangement was used to introduce buffer containing stimulating agents.

[^{3}H]ACh Release from Isolated Retina. Based on a modification of experiments described by Masland and Livingstone (17), uptake of [^{3}H]choline was used to label endogenously synthesized pools of [^{3}H]acetylcholine (ACh). Eye cups from adult rabbits (2.5-3 kg) were incubated in buffer containing 50 µCi of [^{3}H]choline for 20 min at 37°C under flashing light. The tissues were rinsed and retinas isolated as described above. Retinas were post-incubated for 45 min in control buffer without $CaCl_2$ before the release assay. Retinal effluents (10 µl) were collected every 2 min for a period of 40 min before and 20 min after the application of a 20 µl volume of 50 µM kainic acid. Radioactivity contained in the samples was determined by liquid scintillation spectroscopy. Based on results from previous studies (18), greater than 90% of the light-evoked release of radioactivity collected in a similar manner is associated with [^{3}H]ACh. At the end of the assay, the retinas were transferred from the gauze to filter paper by placing the dry filter on top of the wet gauze and then carefully peeling back the gauze. The filters were placed overnight in 1 ml of 1% sodium dodecyl sulfate (SDS) plus 1 mM EDTA before counting. The release rate was calculated as the percent of total radioactivity in the sample released per minute. Comparison were made using the student's t-test.

Extracellular Levels of Endogenous Glutamate. Isolated retinas, collected from adult animals or from pups on postnatal days 1 (P1), 3 (P3), and 5 (P5), were prepared as described previously and placed in the buffer chamber (Fig. 1). Samples of retinal effluent were obtained by extracting 10 µl of surface fluid every 2 min for a total of 10 min before and 20 min after addition of 20 µl buffer containing 500 mM KCl. The bath before stimulation contained 5 mM sucrose; during exposure to KCl, the bath contained 2.5 mM $CaCl_2$. The first five samples were pooled to obtain a 50 µl pre-stimulus collection. Extracellular levels of endogenous glutamate were also collected before and after exposure to 100 µM D-aspartate at postnatal days 1, 5 and adult.

Endogenous Glutamate Content. Retinas were quickly isolated from adult animals and pups on P1, P3 and P5 and frozen immediately on dry ice. Each retina was lysed with 1 ml of cold 50% ethanol. Samples were then homogenized by hand and centrifuged for 30 min. The pellet was discarded and the supernatant was stored at −80°C for analysis by gas chromatography/mass spectroscopy. The samples were lyophilized and derivatized following the addition of 100 ng of deuterated glutamate as an internal standard. Using thionyl chloride, methyl alcohol, and heptafluorobutyric anhydride in the derivatization process, the methyl ester derivative was analyzed on the Finnigan Incos 50. Compounds were separated using gas chromatography (capillary column from J&W; DB5, 30m, film thickness of µm). The amount of glutamate was calculated using the ratio of peak intensities of the internal standard to that of sample.

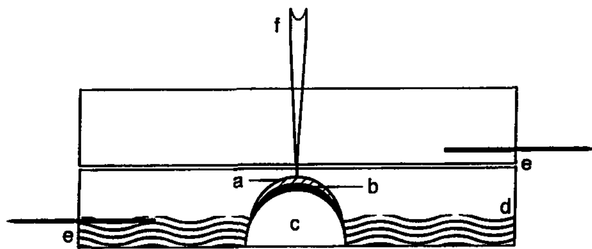

Fig. 1. Superfusion apparatus. Isolated, whole retina (**a**) is placed on gauze (**b**) on top of plastic dome (**c**) in the chamber. Gauze is used to wick buffer to the retina. The level of the buffer reaches the gauze without submersing the retina. The buffer level (**d**) is maintained by inlet and outlet valves (**e**). Ten µl samples are collected manually from the surface of the retina using a microcapillary pipette (**f**).

RESULTS

Release of [^{3}H]Acetylcholine. A preparation designed to measure extracellular levels of neurotransmitters in brain slices under basal or stimulated conditions (16) was adapted for use with isolated, whole retina. [^{3}H]ACh was used as a test compound in order to establish the physiological parameters of the new preparation. The extracellular levels of endogenously synthesisized [^{3}H]ACh has been well-characterized using *in situ* eye cup preparations (18). These investigations demonstrated the endogenous synthesis of [^{3}H]ACh from [^{3}H]choline and its subsequent basal and stimulated release in adult rabbit retina. Using a modification of the protocol reported by Massey and Redburn (18), isolated retinal eye cups were incubated under flashing light in [^{3}H]-choline for 20 minutes in order to allow uptake of the isotope and conversion to [^{3}H]ACh. After post incubation, retinas were removed and placed on gauze in an incubation chamber. Ten µl samples of surface fluid were collected from the exposed outer surface of the retina at 2 minute intervals (Fig. 2A).

Under basal conditions 2.8% (±0.52 SEM) of the total radioactivity present in the retina was recovered in each 10 min sample (Fig. 2B). The addition of 20 µl of 50 µM kainic acid caused a doubling of this amount to 6.8% per 10 min. The stimulation was first detected 6 minutes after addition of kainic acid with peak rates occurring 16 minutes later (Fig. 2A). In all cases the amount of radioactivity remaining in the retina at the end of the collection period was greater than 50% of the amount present at the beginning of the superfusion. These results compare favorably to those obtained with in situ eye cup and thus offer validation of the new preparation employed.

Extracellular Levels of Endogenous Glutamate. The amount of endogenous glutamate released from neonatal

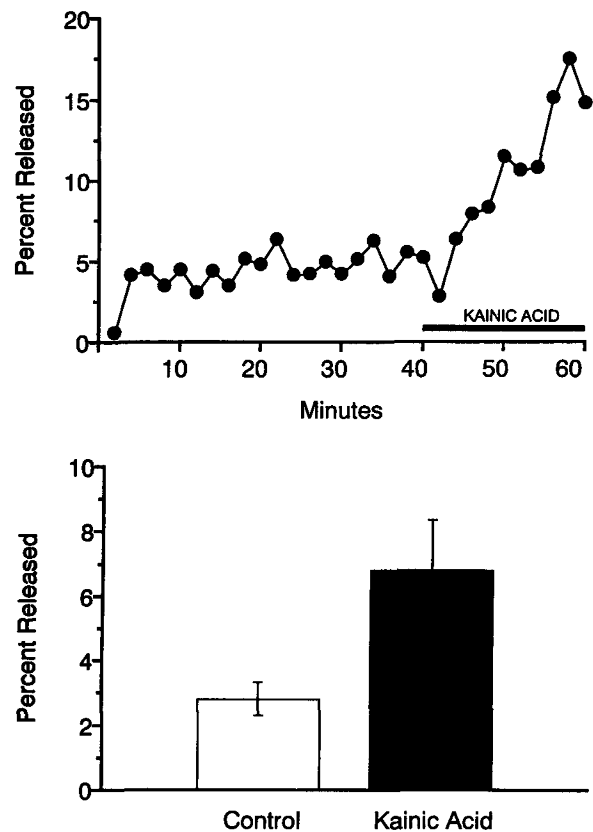

Fig. 2. Release of [^{3}H]acetylcholine. A) Level of [^{3}H]ACh in individual superfusion samples from a representative experiment. Collections were made at 2 min intervals for 40 min in control buffer followed by an additional 20 min collection period in the presence 50 µM kainic acid. **B**) Average level of [^{3}H]-ACh in superfusion samples exposed to control buffer or buffer containing kainic acid, measured as an average of 20 responses over 40 min. Values represent the DPM in the collection sample/total DPM present in the tissue. Mean ± SEM; n = 5 rabbits.

neurons under basal and stimulated conditions was examined by measuring quantities present in the retinal surface fluid collected from the superfusion apparatus described above. The extracellular levels of glutamate in control superfusates were higher at P1 (260 ng/50 µl) than at the other developmental ages examined (Fig. 3; white bars). At P3 and P5, levels were approximately fourfold lower; in the adult they were one third lower than in the neonate. The amount of glutamate present in control samples collected sequentially remained fairly constant over a period of 20 min.

To determine if extracellular glutamate concentrations could be increased by depolarization-stimulated release, we exposed superfused retinas to a 20 µl volume of 500 mM KCl. The amount of glutamate in samples collected under stimulated conditions (Fig. 3; black bars)

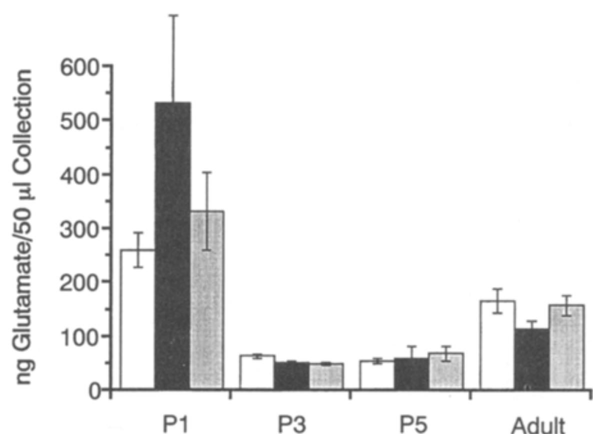

Fig. 3. Extracellular levels of glutamate in developing retina. White bars represent basal levels collected in five 10 µl samples over 10 min. Black bars represent levels of extracellular glutamate samples collected in the 10 min period immediately after application of 20 µl of 500 mM KCl. Gray bars show the levels of glutamate in samples collected during the 10 to 20 min interval after KCl stimulation. Means ± SEM, n = 3 rabbits for each age.

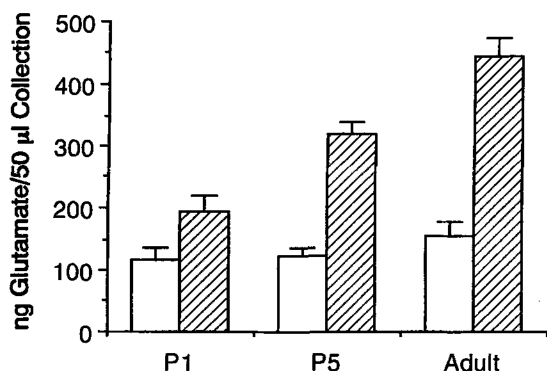

Fig. 4. Extracellular levels of endogenous glutamate after exposure to 100 µM D-aspartate. White bars reflect basal efflux of glutamate in five 10 µl samples collected over 10 min. Hatched bars represent the basal efflux of glutamate after addition of 100 µM D-aspartate. Means ± SEM, n = 5 rabbits for each age.

at P1 was roughly twice that seen before stimulation (532 ng/50 µl). The releasable pool was approximately 9 times larger than that seen at P3 and P5; it was 3.3 times larger than that seen in the adult. The only increase in extracellular levels in adult tissue was a small and somewhat delayed response observed in the collections begun 10 minutes after stimulation (Fig. 3; gray bars).

Extracellular Levels of Endogenous Glutamate in the Presence of D-Aspartate. The intent of these experiments was to test whether the higher levels of extracellular glutamate observed under basal and stimulated conditions in the neonate were due in part to a lack of glial (Müller) cell uptake and metabolism at early ages.

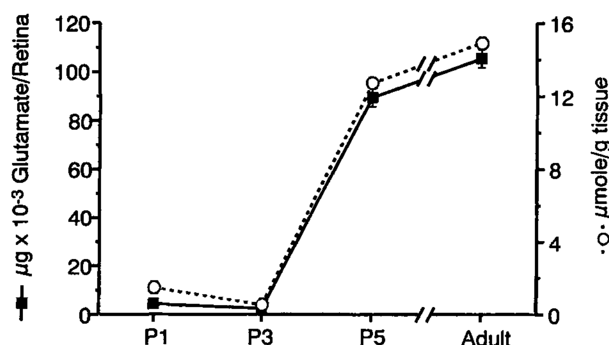

Fig. 5. Total content of endogenous glutamate in developing retina. Values represent total glutamate per retina (ng) and amount of glutamate per g weight (µmoles/g tissue). Means ± SEM, n = 4-6 rabbits at postnatal days (1, 3, 5 and adult).

D-aspartate is a competitive inhibitor of neuronal and glial uptake sites for glutamate. Any increases in levels of extracellular glutamate after addition of D-aspartate can be attributed to a block in the re-uptake of previously released glutamate. A direct relationship should exist between the size of the D-aspartate response and the level of uptake activity.

The effect of D-aspartate on extracellular glutamate levels in retina was as predicted. The basal level of extracellular glutamate increased in the presence of D-aspartate at all ages (Fig. 4). However, the increase was largest in the adult, moderate at P5, and modest at P1. The level in the adult retina after D-aspartate (446 ng/10 min) was approximately 2.9 fold higher than in control buffer (156 ng/10 min). The amount of glutamate in P5 samples collected in the presence of D-aspartate (318 ng/10 min) was significantly larger than at P1 (194 ng/10 min). These data suggests that glutamate uptake activity is lower in neonates and thus is a contributing factor to the high extracellular levels observed.

Total Endogenous Glutamate Content. Total endogenous stores of glutamate were measured by GC/MS over the course of post natal development. Results were calculated as µmoles glutamate/g wet weight of tissue and as µg of glutamate/retina. Small but measurable pools of endogenous glutamate were extracted at birth (1.46 µmole/g tissue, Fig. 5). The largest increase in the size of the endogenous pool occurred between days 3 and 5 which is consistent with the peak period of synaptogenesis. The retina contained as much as 89.3 µg glutamate at P5. Although a significant increase in the total content of glutamate occurred between P5 and the adult (12.7 vs. 14.9 µmol per g tissue), the rate of increase was less than the differential between P3 and P5. The concentration as well as total amount of glutamate

per retina exhibited similar patterns of increase during development.

DISCUSSION

Characterization of the Release Assay. In the present study, a technique designed to monitor release of neurotransmitters from brain slices (16) has been adapted for use in the retina. This preparation provides a direct measure of the amount of endogenous neurotransmitter found in the extracellular fluid of the isolated, whole retina (19). After removal from the eye cup, the retina is placed on a gauze-covered pedestal. The retina is kept moist by the wicking action of the gauze which absorbs buffer from the incubation chamber, and retinal effluent is collected from the exposed photoreceptor surface with a microcapillary pipette. With this arrangement, samples of effluent can be collected from the retinal surface with minimal dilution from buffer thus providing an advantage for sensitive measurements of endogenous transmitters.

In contrast to previously used procedures which collected samples from the vitreal surface, collections in the current system are made from the outer retinal surface which has been mechanically separated from the RPE to create an open subretinal space. In addition to allowing access into the subretinal space, this procedure also disrupts the outer limiting membrane which may decrease Müller cell uptake of glutamate at the scleral surface of the retina. Furthermore, the uptake and metabolism of glutamate may be associated with the expansive Müller cell footpads in the inner retina. For these reasons, it is likely that the effluent in this system preferentially contains more of the substances released from the outer retina.

One potential disadvantage of this system is that the actual area of the retina which contributes to the measured release is unknown allowing only relative calculations of the amount released/mg retina. In addition, the introduction of stimulants through a micropipette exposes the retina to concentrations gradients as opposed to the uniform concentrations achieved in a submersed preparation. The area nearest to the pipette receives the highest exposure to the exogenously applied substances while areas further away receive a lesser exposure. The sampling area may express a range of responses to different concentrations of the stimulus. While these theoretical concerns should be kept in mind, they do not appear to have a major effect on actual results, based on the comparative tests described below.

[^{3}H]Acetylcholine Release. To test the new preparation, measurements of [^{3}H]ACh release were compared with those obtained with an in vivo eye cup preparation first described by Massey and Neal (20). In both preparations, [^{3}H]choline is taken up by the retina, converted to [^{3}H]ACh in the presence of flashing light, and released upon stimulation (17,18).

Similarly, the response to kainic acid stimulation was roughly equivalent in the two different preparations. This peak response measured in the in vivo eye cup was greater than that seen in the isolated retina, but this difference can reasonably be attributed to the shorter collection periods attainable in the eye cup. When results are averaged over a 20 min collection period, both preparations showed an approximate doubling of the basal release rated after a pulse of kainic acid. A 2.5 fold increase was observed in the eye cup [recalculated from Linn et al., (21)] compared to a 2.1 fold increase in the isolated retina. It should be kept in mind that the similarity in results is observed in spite of the major differences in characteristics of the two assay systems. In the eye cup, the measurements reflect the average response from virtually all of the retina since the buffer which bathes most of the vitreal surface is used to introduce the chemical stimulant (kainic acid) and to provide samples for monitoring released [^{3}H]ACh. In the isolated retina preparation, a micropipette is used for introducing and removing surface fluid along the outer retinal border. In this preparation, only a fraction of the retina will actually receive stimulating concentrations of kainic acid and in turn contribute to the released [^{3}H]ACh. Since the general properties of release as well as the actual stimulation rates were similar in the two assay systems, we assume that the release from this fraction of isolated retina is reflective of averaged responses from whole retina and is therefore a valid measure of neurotransmitter released into the extracellular space.

Extracellular Levels of Endogenous Glutamate in Developing Retina. One of the most significant and unexpected findings of this study was the relatively high levels of extracellular glutamate measured in neonatal retina. Under all three conditions examined (unstimulated, immediately after potassium stimulation, and 10 min after stimulation), the amount of glutamate present in retinal superfusates at birth was greater than at any other age. The concentration in superfusion samples ranged from 30–70 μM, levels which have been shown to be toxic in adult retina (22,23). Even so, these values represent a minimal range for the true concentration which must exist within the extracellular space of neonates because the collection sample is diluted by buffer during collection. Because the absolute amount of dilu-

tion is not known, the precise glutamate concentration cannot be calculated.

Based on studies of glutamate immunoreactivity (14) the source of extracellular glutamate appears to be from cell bodies and processes of maturing glutamatergic neurons. Heaviest staining was observed in the ganglion cell layer and in the inner plexiform layer at birth. Occasional amacrine cells were also labeled at the distal border of the INL. In the outer retina staining was observed in the position of cell bodies of developing cone photoreceptors and horizontal cells and their processes within the OPL. Labeling of cones increased during development, coincident with the period of synaptogenesis between P1 and P5 (24). In contrast, horizontal cells lost their immunoreactivity to glutamate by P5, along with various other markers for the GABAergic phenotype, suggesting that the glutamate pool in horizontal cells may be used for GABA synthesis. Nevertheless, we assume that all of the glutamate immunoreactive cell classes are the potential sources for extracellular glutamate in the neonate. Glutamate appears to be released spontaneously and in a depolarization-stimulated manner, since addition of potassium caused a significant increase in extracellular levels. Few synapses of any kind are present in the retina at birth, thus it is likely that glutamate is released from non-synaptic regions, including cell bodies, neurites and growth cones. Basal and potassium stimulated release of neurotransmitters from growth cones has been previously demonstrated in other parts of brain (25,26).

The decrease in extracellular glutamate cannot be due to a decrease in overall glutamate concentrations. The total endogenous content of glutamate increases dramatically after postnatal day 3 approaching adult levels by P5. The increase in glutamatergic pools between day 3 and 5 occurs in synchrony with the reported generation and synaptogenesis of rod photoreceptors and bipolar cells which constitute two of the largest classes of neurons in the rabbit retina (24).

Similar patterns of increase were noted in both the total amount of glutamate per retina (ng/retina) and in the concentration of glutamate (μmoles/g tissue). We conclude that not only are glutamatergic cells being generated during this period but that the concentration of glutamate within individual cells is increasing as well.

The Role of Uptake Systems in Developmental Regulation of Extracellular Glutamate Levels. It seems unlikely that the decrease in the extracellular glutamatergic milieu during development reflects an actual decrease in glutamate release. A more plausible explanation is that the maturation of glutamate uptake systems may be somewhat delayed compared to the functional expression of non-synaptic release.

At least two uptake systems are known to exist in retina, each with distinctive developmental characteristics. First, adult Müller cells exhibit avid uptake and metabolism of glutamate (27,28). However at P1, autoradiographic analysis of [^{3}H]glutamate uptake shows that Müller cells are immature and uptake rates are limited (29). In addition, Redburn et al. (14) have analyzed [^{3}H]—metabolites using TLC and demonstrated that the metabolic conversion of [^{3}H]glutamate to [^{3}H]glutamine occurs at a very slow rate at P1. Thus the inactivity of Müller cells at birth may be the major permissive factor in allowing accumulation of high levels of glutamate in the extracellular compartment at birth. The observed decrease in extracellular glutamate pools by P3 may reflect the onset of Müller cell uptake activity.

A second uptake system for glutamate is present in retinal neurons. Although arguably less significant than Müller cell activity, the uptake and metabolism of glutamate by the large number of bipolar cells and rod photoreceptors generated by P5 may further decrease the levels of extracellular glutamate between P3 and P5.

The observed effects of D-aspartate further support the proposed role of uptake as a major determinant of extracellular glutamate levels. In the retina, D-aspartate serves as a competitive inhibitor for the uptake of glutamate into both neuronal and glial cells where it is broken down to glutamine (30,31). In the presence of a blocker of the glutamate uptake site, such as D-aspartate, glutamate is released from the affected cells but cannot be taken back up thus creating an accumulation of the neurotransmitter in the extracellular compartment. If addition of D-aspartate causes an increase in extracellular glutamate, then it can be assumed that uptake systems are normally active under control conditions.

Our results show that the basal level of extracellular glutamate in the presence of D-aspartate is higher at all ages: P1, P5, and adult. However, the increase is modest in the neonatal retina, larger at P5, and greatest in the adult. Since D-aspartate has significantly less effect at birth, we conclude that overall uptake activity at this age is limited and thus is a likely contributory factor to the high extracellular levels of glutamate.

In conclusion, these results raise interesting questions regarding the role glutamate may play in neuronal development. Putative roles suggested by other investigators' include programmed cell death, dendritic pruning, axon elongation and neurite sprouting (6,32,33). The selective survival of developing cell populations in the presence of high glutamate levels may rely on the regulation of glutamate receptor subtypes and associated

second messenger systems. Our data suggests that the maturational state of retinal glia is likely to be a key factor in regulation of whatever developmental signal may be transmitted by glutamate.

ACKNOWLEDGMENTS

We would like to acknowledge Ms. Cheryl K. Mitchell for excellent technical assistance and help in preparation of the manuscript, and Dr. Bill Seifert from the Analytical Chemistry Center for invaluable assistance in analyzing samples by gas chromatography/mass spectroscopy. Dianna A. Redburn is a Jules and Doris Stein Research to Prevent Blindness Professor. These studies were supported in part by NEI grants EYO 1655, EYI 0608 and EYO 07024 to D. A. R. and a Fight for Sight Fellowship from the National Society to Prevent Blindness in memory of James Thurber to M. F. H.

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