Developmental Changes in Neurotransmitter Receptor Binding in the Human Periaqueductal Gray

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Abstract. The periaqueductal gray (PAG) plays a central role in the integration of defense responses to threatening or stressful stimuli. Little is known about the neurochemical development of the human PAG around the time of birth, when the fetus makes the transition to extraterine life and independent defense responses are needed. We analyzed receptor binding to selected neurotransmitters implicated in PAG function in 7 fetuses (19 to 26 gestational weeks), 9 infants (38 to 74 postconceptional weeks), 1 child (4 years), and 3 adults (20 to 68 years). Tissue autoradiography was used with radioligands for opioid, nicotinic, muscarinic, kainate, and serotoninergic receptors. By midgestation, binding to nicotinic, muscarinic, serotoninergic, opioid, and kainate receptors is already localized in the human PAG. The subsequent developmental profiles are unique for each radioligand. Binding to nicotinic and serotoninergic receptors decreases significantly from the fetal to mature periods, but at different tempos. In contrast, there is no significant change from midgestation to infancy for muscarinic, kainate, and opioid binding; between infancy and the mature period there is a downward trend in binding for muscarinic and kainate receptors and an upward trend for opioid receptors. This study provides baseline information about the neurochemical development of the human PAG in early life. This information is of value in considering the neurochemical substrate of the maturation of defense responses in human infancy, and in evaluating potential neurochemical disorders of the developing human PAG.

Key Words: Human brain development; Kainate receptor; Muscarinic receptor; Nicotinic receptor; Opioid receptor; Serotoninergic receptors.

INTRODUCTION

Based upon extensive animal studies, the midbrain periaqueductal gray (PAG) is postulated to play a central role in the integration of responses to threatening or stressful stimuli (1–3). This integrative role is based in part upon the recognition of multiple and diverse functions of the PAG which are vital components of the defense response, i.e. fear, anxiety, analgesia, posturing, vocalization, and cardiovascular and respiratory adjustments (4–9). The PAG is interconnected with rostral limbic regions and brainstem-spinal cord sites which mediate pain, sympathetic autonomic control, vocalization, and somatic motor responses to threatening stimuli (2). Several neurotransmitters are implicated in these functions, including acetylcholine (ACh), glutamate, serotonin (5HT), and opioids (10–15), and these and other transmitter-specific cell bodies, receptors, and/or terminals have been mapped within the PAG in animal studies (14, 16–19). Virtually nothing is known, however, about the chemoarchitecture of the human PAG, especially around the time of birth, when the fetus makes the transition to extraterine life and independent defense responses are needed. In the following study, we determined the quantitative distribution of receptors to selected neurotransmitters in the developing human PAG with tissue receptor autoradiography. The purpose of the study was to determine the patterns of binding of muscarinic and nicotinic cholinergic, opioid, kainate, and serotoninergic receptors in the human PAG from midgestation through early infancy, and to compare these patterns to those in the mature brainstem. The radioligands were intentionally selected to be broad and to bind to multiple subtypes of each receptor class, because of the paucity of available information about receptors in general in the developing human brainstem.

MATERIALS AND METHODS

Tissue preparation. The specimens analyzed in this study are part of a database under development in our laboratory for delineating brainstem chemical anatomy in early human life (20–24). Brainstems were obtained from individuals with postmortem intervals ≤24 hours (h). Age is expressed as postconceptual (gestational plus postnatal) weeks. The fetal specimens at midgestation were obtained from elective abortions autopsied in the Department of Pathology, Brigham and Women's Hospital, Boston, MA, with approval of the Clinical Protection Committee. Fetal age was determined by foot length measurements (25).

Binding studies and generation of brainstem autoradiograms. Quantitative receptor autoradiography was performed in frozen human midbrains based upon methods developed in experimental animals and/or human postmortem tissues (Table 1)(20–24, 26, 27). Our procedures for tissue preparation and sampling have been described in detail (20). Midbrain sections from each
case were incubated with different radioligands for comparison of muscarinic cholinergic, nicotinic cholinergic, opioid, serotoninergic (5HT), and kainate (KA) receptor binding patterns. In a sample of adjacent serial sections from each case, the following radioligands were applied: {\(^3\text{H}\)quinuclidinyl benzilate (QNB) for muscarinic cholinergic receptor (mACHR) binding (22, 26); {\(^3\text{H}\)nicotine for nicotinic cholinergic receptor (nACHR) binding (21); {\(^3\text{H}\)naloxone for opioid binding (20); {\(^3\text{H}\)lysergic acid diethylamide for 5HT binding (24, 27); and {\(^3\text{H}\)KA for KA binding (23) (Table 1). Nonspecific binding was determined with appropriate displacer (Table 1). Our procedures for washing and drying the sections and generating the autoradiographs have been previously described (20, 21). Sections were exposed to \(^3\text{H}\)-sensitive film (LKB Ultratfilm\(^\text{\textregistered}\)) for 8 to 24 weeks (Table 1).

Quantitative analysis of brainstem autoradiograms. For the quantitative analysis of neurotransmitter receptor binding in the developing human PAG, two sections from a precisely defined level were analyzed from each case. The atlas of Olszewski and Baxter was used as reference (28). The level sampled is defined with its atlas plate number: caudal midbrain, level of the decussation of the superior cerebellar peduncle (Plate XXII). In order to define the anatomic boundaries of brainstem nuclei, the tissue sections which generated the autoradiograms were stained with cresyl violet or hematoxylin-and-eosin and compared with the autoradiogram. Of note, this level analyzed in this study includes only the caudal PAG. Due to lack of tissue availability, we were not able to analyze the rostral PAG at the level of the red nucleus.

Quantitative densitometry of autoradiographs was performed with a MCID imaging system (Imaging Research Inc., Ontario). Optical densities were converted to specific activities in femtomoles/milligram (fmoles/mg) tissue with \(^3\text{H}\)-standards. Receptor density was determined in specific nuclei by digitizing the nuclear boundaries directly upon the specific activity mosaics displayed on the color monitor, with superimposition of the cell-stained tissue section over the specific activity mosaic, when necessary, with software available on the imaging system. Specific activity measurements were made in a blinded fashion, without knowledge of clinical diagnosis or postconceptional age. Specific activity data are displayed as computer-generated mosaics with a linear, 15-step color scale. The range varies depending upon the specific radioligand used. Mean binding levels among the 3 groups (fetal, infant, and mature) were compared using the one-way analysis of variance. Pairwise comparisons of the means were conducted using the Tukey method in order to control the Type I error rate of the multiple comparisons (29). A p value less that 0.05 was considered significant.

### RESULTS

Clinicopathologic Information. We examined neurotransmitter receptor binding in the developing human PAG in a total of 19 cases. The midbrains of 7 midgestational fetuses (median: 22 postconceptional weeks [wk]; range 19 to 26 wk) and 9 infants (median: 52 postconceptional wk; range 38 to 74 postconceptional wk) were examined. The "mature" midbrains of 1 child (4 years [y]) and 3 adults (20 to 68 y) were analyzed as...
indices of maturity. The 9 infants died of acute illnesses without terminal mechanical ventilation, and without clinical neurological dysfunction. The median postmortem interval for all the cases in the study was 7 h (range 1 to 21 h), with a median of 3 h for the fetal cases, 7.5 h for the infant cases, and 7 h for the mature cases. There was no apparent effect of postmortem interval upon neurotransmitter receptors binding in the PAG (20–24).

Developmental Changes in Neurotransmitter Receptor Binding in the Developing PAG. The developmental profiles of neurotransmitter receptor binding were unique for each of the 5 radioligands analyzed (Figs. 1A, 2A). For [3H]-LSD binding to serotoninergic receptors, there was a significant difference among the fetal, infant, and mature periods (p=0.001) (Figs. 1A, 2A) (Table 2). Binding decreased dramatically between the fetal and infant periods (47%), and between the infant and mature periods (59%) (Fig. 2A). For [3H]-nicotine binding to nicotinic receptors, there was also a significant difference among the fetal, infant, and mature periods (p=0.009) (Figs. 1B, 2B) (Table 2). Binding decreased dramatically between the fetal and infant periods (54%), but, unlike the 5HT receptors, remained relatively unchanged between the infant and mature periods. For [3H]-naloxone binding to opioid receptors, there was only a marginally significant difference among the fetal, infant, and mature periods (p=0.08) (Figs. 1D, 2D) (Table 2). Binding was relatively constant between the fetal and infant periods, and increased slightly (19%) between the infant and mature periods. For [3H]-QNB binding to muscarinic cholinergic receptors, the levels were relatively constant across the fetal, infant, and mature periods, and there were no significant differences among the age groups (Figs. 1C, 2C) (Table 2). For [3H]-kainate binding, the levels tended to decrease with maturation, but the changes were not significant among the fetal, infant, and mature periods (Figs. 1E, 2E) (Table 2). This lack of significance may reflect the small sample size and notable variation of KA binding among cases. Overall, between the fetal and infant period, the most dramatic changes occurred in the serotonergic and nicotinic cholinergic receptor binding with 47% and 54% decreases, respectively. Opioid, muscarinic cholinergic, and kainate binding remained relatively constant within the same fetal to infant period. Between the infant and mature period, the most dramatic changes were in the serotoninergic binding, with a 59% decrease; kainate and nicotinic cholinergic binding remained the same, muscarinic binding tended to decrease, and opioid binding increased slightly with marginal significance.

Subdivisions within the Human PAG. Upon examination of autoradiographic films, there were no obvious subdivisions of neurotransmitter receptor binding in the caudal level of the PAG analyzed. The only visually distinct subdivision of binding was in the kainate receptor where a ring of high binding appeared adjacent to the aqueduct of Sylvius (Fig. 1E).

DISCUSSION

The PAG is a structurally, chemically, and physiologically complex region that is involved in the integration of defense responses to threatening or stressful stimuli (1–3). In the current study, we found that the chemarchitecture of the PAG in humans is likewise complex and varies dramatically across development. By midgestation, binding to nicotinic, muscarinic, serotoninergic, opioid, and kainate receptors is already localized in the human PAG. The subsequent developmental profiles are unique for each radioligand. Binding to nicotinic and 5HT receptors decreases significantly from the fetal to mature periods, but at different tempos. In contrast, there is no significant change from midgestation to infancy for muscarinic, kainate, and opioid binding; between infancy and the mature period there is a downward trend in binding for muscarinic and kainate receptors and an upward trend for opioid receptors. In the following discussion, we consider factors underlying the different neurotransmitter receptor binding profiles, and we discuss the issue of neurochemical heterogeneity in the human PAG in relationship to our data.

The most dramatic changes we found in neurotransmitter receptor binding were decreases in 5HT and nicotinic binding between the fetal and infant periods, and decreases in 5HT binding between the fetal and mature periods. In the mature PAG, 5HT and nicotinic binding were virtually negligible. Reduction in neuronal density and/or progressive tritium quenching are underlying mechanisms which can account for the reductions in neurotransmitter binding during development. If a reduction in neuronal density were solely responsible for decreased 5HT and nicotinic binding, then we would expect decreased binding in all 5 radioligands sampled: since opioid, muscarinic, and kainate binding remained essentially unchanged between the fetal and infant periods, there must be other contributing factors. Progressive tritium quenching by lipids (myelination) is also unlikely to provide sole explanation for the decreased binding. In previous work, we found that in well-circumscribed nuclei not heavily myelinated, the effect of quench between the fetal and infant periods is negligible (21–24). Since the developing PAG is not heavily intermixed with myelinated fibers (28), quench is not likely to contribute significantly to decreased binding in this region as well.

Other possible explanations for decreasing binding with increasing age are a developmentally programmed loss of dendritic spines with postsynaptic receptors, loss of receptors themselves, and/or decreased receptor affinity. Given that there are multiple subtypes of serotoninergic and nicotinic receptors, and that the ligands that we used label more than one subtype, the decline in binding...
Fig. 1. The relative distribution of neurotransmitter receptors in color-coded, specific activity mosaics across development in the human periaqueductal gray. The color scale with specific activity levels in fmole/mg tissue is shown. A. Development of [3H]-LSD (lysergic acid diethylamide) binding to serotoninergic receptors. B. Development of [3H]-nicotine binding to nicotinic cholinergic receptors. C. Development of [3H]-QNB (quinuclidinyl benzilate) binding to muscarinic cholinergic receptors. D. Development of [3H]-naloxone binding to opioid receptors. E. Development of [3H]-kainate binding to KA receptors. Icol = inferior colliculus; MLF = medial longitudinal fasciculus; RD = N. raphe dorsalis.
Fig. 1. Continued.
Fig. 2. A mean (with standard error of the mean) of specific activity of neurotransmitter receptor binding is compared among the fetal, infant, and mature groups. A. [H]-LSD-binding to serotoninergic receptor (5HT) decreases across development. B. [H]-Nicotine binding (NIC) to nicotinic cholinergic binding decreases from the fetal to infant period. C. [H]-QNB binding to muscarinic cholinergic receptors (MUS) stays relatively constant throughout development with a slight decrease between the infant and mature period. D. [H]-Naloxone binding to opioid receptors (OP1) stays relatively constant throughout development with a slight increase in binding from the infant to mature period. E. [H]-Kainate binding to KA receptors is relatively constant across development with a slight decrease in binding from the infant to mature period.

may also represent differential rates of decline in expression among subtypes. A final consideration is that 5HT and nicotinic receptors in particular are high in the fetal period because they subserve trophic effects of serotonin and acetylcholine, respectively; once this function is complete, a programmed loss of receptors occurs such that only receptors needed for neurotransmission are retained in the mature structure. This idea is based on strong experimental evidence that serotonin influences neuronal development via 5HT-receptor mediated mechanisms early in development (30, 31), and acetylcholine effects neuronal process outgrowth via nicotinic-receptor mediated mechanisms (32). Parenthetically, nicotine agonists injected into the adult PAG produce flight reactions (11), and 5HT receptors mediate inhibition (15, 33). Further study is needed to determine the precise mechanisms that account for the decreased 5HT and nicotinic binding across early development in the human PAG.
NEUROTRANSMITTER RECEPTOR BINDING IN DEVELOPING HUMAN PERIAQUEDUCTAL GRAY

TABLE 2
Receptor Binding in the Human PAG

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Fetus*</th>
<th>Infant*</th>
<th>Adult*</th>
<th>3-group p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin</td>
<td>111 (10)</td>
<td>59 (5)</td>
<td>24 (7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Nicotinic</td>
<td>29 (4)</td>
<td>13 (2)</td>
<td>12 (4)</td>
<td>0.009</td>
</tr>
<tr>
<td>Muscarinic</td>
<td>162 (27)</td>
<td>176 (20)</td>
<td>114 (22)</td>
<td>NS</td>
</tr>
<tr>
<td>Opioid</td>
<td>19 (1)</td>
<td>21 (1)</td>
<td>26 (2)</td>
<td>0.080*</td>
</tr>
<tr>
<td>Kainate</td>
<td>14 (3)</td>
<td>11 (3)</td>
<td>7 (2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Legend: See Table 1 for radioligands used; +, measurements in fmol/mg (±SEM); NS, nonsignificant; *, marginally significant (0.05 < p < 0.10); n, sample size.

A major observation of this study is the lack of visually distinct subdivisions within the caudal human PAG as defined by neurotransmitter receptor autoradiography. Although there has been controversy over the existence of cytoarchitectonic subdivisions (49), there have been many animal studies in their support (50–52). Olszewski and Baxter defined 3 cytoarchitectonic subdivisions within the human PAG: nucleus dorsalis, nucleus lateralis, and nucleus medialis (28). In this study, we did not find a heterogenous distribution of receptor binding between the medial and lateral portions of the caudal PAG for any of the radioligands analyzed at any age.

Recently, it has been suggested that the PAG is organized in longitudinal columns along the rostrocaudal axis, and that these columns have anatomic and functional specificity (1, 3). While this concept is debated, 4 columnar subdivisions (dorsomedial, dorsolateral, lateral, and ventrolateral) are based upon evidence in animals that forebrain, brainstem, and spinal cord afferents and efferents are arranged longitudinally (53). The dorsolateral and dorsomedial columns receive cortical, subcortical, and a few dorsal horn and trigeminal inputs (54, 55). The lateral column projects largely to the ventromedial, ventrolateral, and dorsal medulla (56–58). Afferents to the lateral column of the intermediate PAG project from the anterior hypothalamic/medial preoptic region, central nucleus of the amygdala, and anterior cingulate cortex (54, 55). Based on animal studies, the dorsolateral column has high densities of muscarinic, kainate, and GABA<sub>B</sub>/benzodiazepine binding sites as compared to the other columns (59). Microinjections of excitatory amino acids into the lateral neuronal column of the intermediate PAG result in an increase in sympathetic and somatomotor activity associated with defensive behavior (1, 56); injections into the ventrolateral PAG evoke opposite effects (1, 56, 58, 60). In the cat PAG, there are subtle differences in the binding of muscarinic, kainate, GABA/benzodiazepine, glycine, and adenosine-A<sub>A</sub> receptors among subdivisions (59). The dorsolateral region, for example, has higher concentrations of muscarinic, kainate and benzodiazepine binding sites than the ventrolateral region (59). In the rat the dorsolateral region has the highest density of binding for all of the glutamate receptor subtypes (19). In the current study, we did not find a heterogenous pattern in the opiate, nicotinic, muscarinic, kainate, and serotoninergic binding in the human caudal PAG during development or maturity. There are possible explanations for this finding. First, in terms of PAG columnar organization, the dorsolateral column is the most distinct column neurochemically (59). It can be distinguished from the lateral and dorsomedial columns, for example, by 3H-QNB and 3H-KA binding in the cat PAG (59). However, these studies have found that the neurochemical specificity of the dorsolateral is most prominent at rostral and
intermediate PAG levels (59). In this study, only the caudal level of the PAG was analyzed, which has a very small size of a dorsolateral column. There is little evidence for neurochemical markers that are unique to the dorsomedial, lateral and ventrolateral PAG columns with the radioligands analyzed in this study. Since the level that was analyzed in this study did not contain a prominent component of the dorsolateral column, it could be expected that there may be a lack of regional difference in binding. Second, neurochemical subdivisions may exist in the human PAG as defined by markers not analyzed in this study, e.g. benzodiazepine binding. Moreover, physiologic subdivisions may exist, although this information is currently unknown. More detailed studies, looking at rostral levels of the developing human PAG, which should contain a larger portion of the dorsomedial column, would be needed to definitively address the issue of PAG neurochemical subdivisions within the developing human PAG. As our study includes a small sample size, it is also possible that subtle neurochemical subdivisions exist but a larger sample is needed to define them.

A potential limitation of this study is that it is based upon an autopsy population of fetuses and infants that died of diverse causes. Although the brainstems were histologically unremarkable, it is possible that the disease or agonal conditions may have adversely affected receptor binding in a particular case, such that its pattern of binding does not reflect that in the healthy living individual. Within the developmental periods examined in this study, the relative distribution of receptor binding was remarkably constant among the cases, irrespective of the cause of death or agonal conditions. This observation suggests a baseline pattern of binding within the time period.

In summary, this study provides baseline information about the neurochemical development of the human PAG in early life. This information is of value in considering the neurochemical substrate of the maturation of defense responses in human infancy, and in evaluating potential neurochemical disorders of the developing human PAG.

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