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Immunohistochemical localization of AMPA-type glutamate receptor subunits in the nucleus of the Edinger-Westphal in embryonic chick

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Abstract

The Edinger-Westphal nucleus (EW) in birds is responsible for the control of pupil constriction, accommodation, and choroidal blood flow. The activation of EW neurons is mediated by the neurotransmitter glutamate, in large part through AMPA-type glutamate receptors (GluRs), whose behavior varies according to the subunit composition. We investigated the developmental expression of the GluR subunits in EW of the chick (*Gallus gallus*) using immunohistochemistry on tissue from embryonic days 10 through 20 (E10–E20). Of the three antibodies used, one recognized the GluR1 subunit, another the GluR4 subunit, and the third recognized a sequence common to GluR2 and GluR3 subunits. No immunolabeling of EW neurons for any GluR subunits was observed prior to E12, although immunolabeling was seen in somatic oculomotor prior to E12. At E12, immunoreactivity for each of the three antibodies was in only approximately 2% of EW neurons. By E14, the abundance of GluR1+ perikarya in EW had increased to 13%, and for GluR2/3 had increased to 48%. The perikaryal abundance of the immunoreactivity for GluR1 and GluR2/3 declined to 3% and 23%, respectively, by E16. At E14, 33% of EW neurons immunolabeled for GluR4, and their frequency increased to 43% by E16, and remained at that approximate percentage through hatching. The increased expression of GluR1 and GluR4 in EW at E14 coincides with the reported onset of the expression of the calcium-binding protein

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parvalbumin, and the calcium currents associated with AMPA receptors formed by these two subunits may play a role in the occurrence of parvalbumin expression.

Keywords

Glutamatergic system; Neurotransmitters; Receptor subunits; Visual pathways; Visual system

The Edinger-Westphal nucleus (EW) is the parasympathetic accessory nucleus of the oculomotor nuclear complex, and in birds it consists of a small group of neurons located dorsolateral to the somatic oculomotor nucleus [11,23,24]. The avian EW provides cholinergic pre-ganglionic parasympathetic input to the ciliary ganglia (CG) of the orbit [11,23], controlling blood flow within choroidal blood vessels [7], pupil constriction, and accommodation of the lens [11]. Neurons of the ciliary ganglion, which possess cholinergic nicotinic receptors [2], are divided into choroidal and ciliary types, which receive inputs from different parts of EW [11,23,24]. The medial EW (EWM) is responsible for innervating choroidal neurons, controlling choroidal vasodilatation, and the lateral EW (EWL) innervates ciliary neurons [11,24], which in turn innervate the muscles of the iris and ciliary body, for pupil constriction and accommodation, respectively [11,23]. Activation of EW neurons appears to be, at least partially, effected by glutamate [27], the major excitatory neurotransmitter of the central nervous system [28].

The neuronal response to glutamate, in general, depends on the type of glutamate receptors present postsynaptically [3,14,28]. Ionotropic glutamate receptors mediate rapid responses, and are constructed from receptor type-specific assemblies of subunits. The alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type receptors are composed of tetrameric combinations of any of four subunits named GluR1, GluR2, GluR3, and GluR4 [14].

The physiological characteristics of AMPA-type GluRs depend on the subunits that constitute the functional receptor [3,14,18,22], with AMPA-type receptors that lack GluR2 being calcium permeable [3,14]. Thus, the depolarization process triggered by AMPA-type receptors lacking GluR2 causes a quick and transient increase in cytosolic calcium [14,28], which is particularly common in neurons showing high-frequency repetitive firing, like EW neurons [8]. Our prior study reported that EW in post-hatch chickens tends to be rich in GluR4 and calcium binding protein parvalbumin but poor in GluR2 [27]. By contrast, adult pigeon EW is rich in GluR1, GluR2/3 and GluR4 immunoreactivity, but poor in parvalbumin. Parvalbumin may be present in EW neurons in chicks to provide buffering for the calcium influx that occurs via their calcium permeable AMPA channels [9,26].

Developmental studies in embryonic chickens have shown that EW neurons are electrophysiologically active at E12, as revealed by cytochrome oxidase activity, and also at that age develop voltage-activated calcium currents [10]. At E14, an increase in parvalbumin occurs in EW neurons, without any further change in voltage-activated calcium currents [8,9,10]. These investigators suggested that the expression of parvalbumin at E14 might occur due to, or in association with, the addition of a calcium conducting channel at E14 [10]. Since the insertion of calcium-permeable AMPA GluR would augment the calcium

conductance in developing EW neurons, we sought to characterize the time course of the expression of AMPA-type GluR subunits within EW in chicken embryos from E10-E21, especially those that form calcium permeable channels (lacking GluR2).

Eighteen chicks (*Gallus gallus*) were used in this study, with three embryos at each of the developmental stages E10, E12, E14, E16, E18 and E20. Embryonic stages were established according to Hamburger and Hamilton [13]. For E10 embryos, the brain was removed and post-fixed by immersion in 4% paraformaldehyde for 24 hours. After E12, embryos were removed from the egg, and anesthetized by an intramuscular injection of ketamine (0.5 mg/10g body weight) and xylazine (0.1 mg/10g body weight). Once anesthetized, E12-E20 embryos were perfused transcardially with buffered saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed, and post-fixed for 5–20 hours in 4 % paraformaldehyde. All fixed tissue was subsequently rinsed with 0.1M PB and cryoprotected for 4–5 days in 30% sucrose in 0.1M PB [26,27]. All animal procedures described were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the University Institutional Ethic Committee.

For the immunohistochemical studies, chick brains were removed from the 30% sucrose PB solution and suspended in tissue freezing medium (TBS, from Triangle Biomedical Sciences, Durham, N.C.). A cryostat (Leica, CM 3050S) was used to cut the avian brains into 18µm sections that were sequentially collected from rostral to caudal onto 0.5 % gelatin-coated (type B from bovine skin, Sigma Chemical Co., St. Louis, MO) glass slides. Each section was sequentially collected on one of four slides. The slide-mounted sections were then dried, and subsequently stored in a –20 C freezer until used for antibody labeling. After removal from the freezer, three of the four series of slide-mounted sections for each chick were brought to room temperature, washed, and the sections outlined with a liquid-repellent slide marker pen (to retain reagents on the sections during the immunostaining procedures). The sections were pre-treated with 0.03 % hydrogen peroxide in 0.1 M PB for 30 minutes, to deplete any endogenous peroxidase. Sections were subsequently rinsed in 0.1 M PB solution and each series incubated overnight at room temperature with one each of three rabbit anti-GluR subunit primary antibodies. The antibodies were diluted in 0.1 M phosphate buffer with 5% normal goat serum and triton 0.3%. We used rabbit antibodies (Chemicon, Temecula, CA, USA) against GluR₁, or GluR₄ (AB1504, lot LV1519215/AB1508, lot LV1462006; respectively), or a GluR2/3 antibody that recognizes an amino acid sequence common to GluR2 and GluR3 (AB1506, lot LV1495573) [28,29]. The primary antibody concentrations were: GluR1 [1:100], GluR2/3 [1:250], and GluR4 [1:100]. According to manufacturer information and published studies, the immunogens for the GluR1, GluR2/3 and GluR 4 antisera show 85% homology to the corresponding avian sequences [6,22]. Western blot analysis in mammals and diverse avian species has shown these antisera selectively detect proteins of the predicted size for each GluR subunit [4,6,20,25,30]. Moreover, blocked controls show the specificity of these antisera in mammals and birds [21], and in the case of GluR2/3 specificity is supported by the concordance in distribution between immunolabeling and GluR2 in situ hybridization [27]. Upon completion of the incubation with primary antibody, the tissue was rinsed in 0.1 M PB

solution. The slide-mounted sections were then incubated in a biotinylated secondary antibody (goat-anti-rabbit, 1:250 diluted in 0.1 M PB, from Jackson ImmunoResearch, West Grove, PA) for 90 minutes at room temperature. The slide-mounted sections were next incubated with Vectastain® ABC solution (Vector Laboratories, Inc., Burlingame, CA) for one hour, rinsed in 0.1 M PB, and the labeling revealed by incubation in a diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, MO) solution (25 mg DAB in 100 ml 0.1 M PB) with 0.03% hydrogen peroxide for five min. The tissue was rinsed in 0.1 M PB, dried on a slide warmer, dehydrated in an ascending alcohol series, coverslipped using Permount®, and examined with a NIKON E-800® microscope [26,27].

To compare the GluR labeling in EW with overall neuronal abundance in EW, the remaining series of slide-mounted sections were warmed to room temperature, and stained with Giemsa, which distinctly labels neuronal cell bodies [15]. For each chicken embryo brain, four series of sections were thus prepared: Giemsa, GluR1, GluR2/3, and GluR4. The Giemsa sections were used to determine the numbers of neurons in EW, which together with counts of immunolabeled neurons, were used to calculate the percentage of EW neurons that were immunopositive. Counting was performed for stages E12 to E20 but not at E10, since no neuronal GluR label was detected at E10. A minimum of ten sections per marker (GluR and Giemsa) for each age group were counted and the values averaged for that age group. Each age group had three animals in it. The following criteria were used to identify Giemsa and GluR positive neurons for counting purposes: the neuronal cell membrane was complete and distinct, the soma was greater than 10 microns and lacked the star-shape characteristic of glial cells, nuclear chromatin was diffuse and even, and the perikaryal cytoplasm was well defined. Note that adult avian EW neurons are over 15µm in size [23]. The Abercrombie correction [1] was used to adjust for double counting. Digital images were captured using a CCD camera and NIH Image software, and figures were prepared using Adobe Photoshop.

EW at stage E10 in embryonic chick can be identified by its clear oval shape (Figure 1). It is located dorsal and slightly lateral to the somatic oculomotor nucleus (OM). At this developmental stage, EW is recognizable but its neurons were less intensely stained for Giemsa than in the adjacent OM (Figure 1). No immunoreactivity for any GluR subunit was observed in EW neurons at E10, although there was some observable neuropil staining. Since EW neurons were not immunolabeled at this age, neuron counts at this age are not reported.

A few EW neurons were distinctly immunopositive for GluR subunits by E12 (Figures 2 and 3), and neuronal abundance for each GluR in EW was approximately 2% (Table 1). The percentage of neurons showing immunoreactivity within embryonic EW dramatically increased for all AMPA subunits by E14: GluR1 (14%), GluR2/3 (48%) and GluR4 (33%) (Table 1). These results can also be observed in the images shown (Figures 2 and 3).

No obvious differences were observed for overall EW neuronal abundance as determined from Giemsa-stained material across developmental stages E12 through E20, but noteworthy stage-related variation was observed for neuronal subunit abundance in EW. For example, the data revealed that GluR1-immunolabeled neurons were more abundant at E14 than the

other developmental stages (Table 1 Figure 3). Similarly, a sharp increase was observed in the percentage of neurons immunolabeled for GluR2/3 at E14, with a slight decline at E16 that was sustained thereafter (Table 1, Figure 3). In the case of GluR4, immunolabeling was less common at E12 than any other age (Table 1 and Figure 3), but then was equally common (30–40%) at all other stages examined.

By E16, the relative numbers of GluR1, GluR2/3, and GluR4 immunolabel had reached the levels observed previously in post-hatch chicks [27] and were unchanged for the remainder of the investigated embryonic period – about 2% for GluR1, 28% for GluR2/3, and 43% for GluR4 (Table 1). Note that the borders of the two nuclear subdivisions (EWM and EWL) were difficult to accurately distinguish in the embryological sections. For this reason, we do not report the developmental timing of GluR immunoreactivity in those specific subdivisions, but rather for EW as a whole.

In embryonic chickens, we have shown here that only approximately 2% of the EW neurons were immunoreactive for GluR1, GluR2/3 or GluR4 at E12. Note that antidromic evoked potentials in response to stimulation of their preganglionic endings in the ciliary ganglion [17], as well as cytochrome oxidase activity [10], can be detected in EW neurons as early as E8, indicating that the EW input to the ciliary ganglion and EW neurons themselves are capable of electrical excitability by E8. Nonetheless, our data indicate that the ability to respond to afferent input via AMPA type glutamate receptors is poorly developed from E8 to E12. For all GluR subunits, the percentage of immunoreactive neurons increased markedly at E14. This is noteworthy because at this developmental stage, Fuji and co-workers reported an appearance of parvalbumin immunoreactivity in EW neurons [10], which would potentially buffer the cytosolic calcium increase caused by the calcium permeable channels. After E14, the frequency of GluR1-immunoreactive neurons dramatically declined to about 2%, and that for GluR2/3 declined to a lesser degree. By contrast, the percentage of neurons immunopositive for GluR4 remained high throughout development, and even seemed to show a slight but steady increase. At E16, five days before hatching, the neuronal frequencies for all GluR subunits had reached the relative neuronal frequencies previously reported in EW for GluR subunits in post-hatch chickens, as determined by immunolabeling and *in situ* hybridization [27]. As reported also for post-hatch chick EW [27], the most prevalent AMPA subunit in embryonic chick EW is GluR4.

The total percentage of EW neurons positive for GluR subunits (i.e. the individual subunit percentages summed together) was less than 100% in both embryonic and post-hatch chicks at each age [27], with the highest total being 93.6% at E14, and a total of 77.1% at E20. This comes somewhat as a surprise, since the primary input to EW is glutamatergic, and AMPA receptors mediate fast glutamatergic neurotransmission – leading to the expectation that all EW neurons should possess at least one type of AMPA subunit. That the data we observed were contrary to this may be explained by either of three possibilities. First, it may be that our percentages are an underestimation of the AMPA receptor occurrence, and reflect a failure to immunolabel all AMPA-possessing EW neurons. Secondly, it may be that we have an overestimation of the total EW neurons labeled using Giemsa, due perhaps to a failure to consistently distinguish neurons and glia. Finally, it may be that not all EW neurons possess

AMPA receptors, and fast glutamate transmission in some is mediated by kainic acid receptors.

With regard to the possibility that our immunolabeling underestimates neuronal GluR frequency in embryonic chick EW, we obtained similar immunolabeling results in post-hatch chick [27]. Moreover, in this current study we did observe robust GluR immunostaining in other regions of the brain, such as the OM neurons that were particularly rich in GluR2/3 and GluR4. Nonetheless, it is possible that for some reason some embryonic chick EW neurons did not bind the antibodies well, and as a result, yield an underestimate of AMPA subunit quantity within EW. In this regard, it should be noted that our *in situ* hybridization data in post-hatch chick also found low levels of GluR2 in chick EW [27].

A possible overestimation of the total number of Giemsa-stained neurons cannot be entirely excluded, since Giemsa can also stain glia. To minimize counting of glia, we used strict criteria to distinguish neurons and glia, as noted in the methods. Moreover, our overall number of Giemsa-stained EW neurons in embryonic chicken is not significantly different than the approximately 1200 neurons reported for adult pigeon EW [23]. Thus, it seems unlikely that an overestimate of EW neurons in chick would account for the low frequency of GluR+ perikarya in EW. Finally, it is possible that another glutamate receptor, kainate (KA), is present in chick EW neurons. KA receptors are abundant in the avian CNS [6,19], and prior studies by others have shown that KA-evoked currents transiently mediate excitatory transmission in chick lumbar motoneurons early in development [19].

Composition of the ionotropic AMPA-type glutamate receptor subunit determines the functional properties of the receptor, including calcium ion permeability [12,14,19]. GluR2 restricts calcium entry through the AMPA receptor channel [12,14,19]. The high percentage of neurons within chick EW that were immunopositive for GluR1 and GluR4 at E14 could result in increased calcium ion entry into these neurons. Fuji and co-workers [10,18] saw an increase in parvalbumin immunoreactivity at E14, yet did not observe a change in voltage-gated calcium currents [10]. They also reported that there was no increase in cytochrome oxidase activity at E14, indicative of no increased mitochondrial activity due to increased neuronal activity [10]. They suggested that the parvalbumin increase might stem from increased intracellular calcium load, attributable to expression of a calcium permeable channel type at E14 other than a voltage-activated calcium channel [10]. The increase in GluR1 and GluR4 subunits at E14 observed here could represent the basis of the increased calcium permeability Fuji and coworkers hypothesized [18]. Parvalbumin is often expressed in neurons that are enriched in these two subunits in many brain regions and in many species [5,16], including pretectal neurons of pigeons [26]. Whether AMPA-mediated calcium entry causes or is merely correlated with the enhanced expression of parvalbumin is uncertain.

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Research Highlights

Glutamatergic inputs control the parasympathetic Edinger-Westphal nucleus (EW).

The EW neurons responses are largely done by AMPA-type receptors.

AMPA receptor subunits are expressed on EW neurons beginning at E12.

AMPA receptor subunits on EW achieve their adult expression and frequency by E16.

Figure 1.

Digital images of coronal sections stained with Giemsa at the level of the nucleus of Edinger-Westphal (EW) in chick at three representative embryonic stages. At E10, in contrast to oculomotor nucleus (OM) that presents clearly stained neurons, neurons are faint in EW, although the boundaries of EW are evident. In general, EW neurons are evident at E12, exhibiting an adult appearance by E16. Scale bar: 300 μ m.

Figure 2.

Digital images of coronal sections that were immunolabeled for GluR1, GluR2/3, and GluR4 at the level of the nucleus of Edinger-Westphal (EW) in chick at three representative embryonic stages. No immunolabeled neurons were observed at E10, however there was some neuropil label. By E14 some scattered EW neurons possessing GluRs were clearly discernible. By E16, the GluR1 immunoreactivity was greatly reduced, and GluR2/3 was reduced to a lesser degree. Square insets at lower left of images show details of the EW area identified by arrows. Scale bar: 300 μ m.

Figure 3.

The relative percentage of neurons immunolabeled for each GluR in EW of chick embryos, from E12 to E20. GluR1 and GluR2/3 were transiently elevated at E14, then reduced after E16 to percentages similar to those reported in post-hatch chicks. GluR4-positive neurons reach their maximum peak of occurrence at E18, and remain at that level. Standard error bars as provided in Table 1 are shown.

Table 1

Percentages of neurons of chick EW according developmental age and marker.

Developmental Age	Total EW Neurons	GluR Type in EW		
		GluR1	GluR2/3	GluR4
E12	983±246	2.7±1.46%	1.6±1.43%	2.3±1.63%
E14	1277±169	13.6±4.34%	46.8±6.42%	33.2±6.06%
E16	1362±145	3.3±1.37%	28.6±4.43%	42.6±5.90%
E18	1289±166	1.3±1.16%	27.4±3.00%	47.7±5.14%
E20	1315±213	1.4±1.01%	25.8±2.99%	46.9±3.27%

The second column shows the mean and standard errors for Giemsa-stained neurons counted in EW for each developmental stage. The third through fifth columns show the percentages and standard errors of neurons in EW immunolabeled for a particular GluR or pair of GluRs. EW neuron counts for Giemsa and GluRs were adjusted according to Abercrombie [1].