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Electrotonic synapses in young rat inferior colliculus

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Summary: Biocytin was injected into neurons in frontal slices of inferior colliculus (IC) from rats. Eighteen out of 76 intracellular injection of biocytin into single neurons resulted in staining of more than one cell (23%). Longer biocytin injection times resulted in a higher incidence and increased number of coupled cells. One minute of biocytin injection resulted in 16% incidence rate of coupling, whereas 5-10 minute of injection resulted in 28%. There was no difference in the incidence of dye coupling when the slice was incubated in the bath for long or short periods after the biocytin injection. However, the dye coupling was correlated with the age of animal; the incidence rate of the coupling among stained cells was 28% in IC slice preparations from 13 day-old rats, whereas the rate was 15% in the 16 day-old ones. These results strongly suggest the presence of electrotonic coupling between neurons of 13-16 day-old rat inferior colliculus.

Key words: Biocytin, electrotonic synapse, inferior colliculus, intracellular staining

Genç sıçanların kollikulus inferiör'ünde bulunan elektriksel kavşaklar

Özet: Sıçanlardan elde edilen kollikulus inferiör'ün ön kesitlerindeki sinir hücrelerine biyosaytin boyası enjekte edildi. Yetmiş altı enjeksiyonun on sekizinde birden fazla hücre boyandı (%23). Enjeksiyon süresi uzadıkça, birden fazla hücre boyanması olayında ve boyanan hücre sayısında da artış gözlendi. Bir dakikalık enjeksiyon %16, buna karşın 5-10 dakikalık enjeksiyon ise %28'lik oranda birden fazla hücre boyanmasıyla sonuçlandı. Biyosaytin injeksiyonundan sonra kesitlerin kayıt odasında kısa veya uzun süreli inkübe edilmesi birden fazla sinir hücresi boyanması oranında herhangi bir fark oluşturmadı. Bununla birlikte, birden fazla hücre boyanması olayı hayvanın yaşı ile ilişkili bulundu. On üç günlük sıçanlardan elde edilen kollikulus inferiör kesitlerinde bu oran %28 iken 16 günlük sıçanlarda %15 olarak saptandı. Bu sonuçlar, sıçan kolliculus inferiör sinir hücreleri arasında elektriksel kavşakların varlığını büyük oranda ortaya koyar.

Anahtar kelimeler: Biyosaytin, elektriksel kavşak, hücre içi boyama, kollikulus inferiör

Introduction

The inferior colliculus (IC) is an auditory nucleus, in which all ascending and descending auditory pathways make an obligatory synapse. It is thought to be involved in integration of binaural sound information. Interactions between neurons in mammalian nervous systems were believed to be mediated exclusively by chemical synapses, but in recent years it has been shown that electrotonic coupling may also exist in a number of adult mammalian brain structures, such as, hippocampus (34), supraoptic neurons (15), striatal neurons (6,16,24,28).

There are several ways to demonstrate existence of gap-junctions, including electrophysiological techniques, e.g. simultaneous recordings from pairs of cells to test directly for electrical continuity (20), visualization of gap junctions, morphological correlates of electrotonic coupling, using either transmission electron microscopy (TEM), freeze-fracture techniques (5), or immunoreactivity to gap junctions (3) or demonstration of dye transfer between coupled cells through gap junctions, namely, dye coupling. The dye coupling method using dyes with low molecular weight (<1000 Dalton), e.g., Lucifer yellow (33) and biocytin (12) has provided a particularly successful method and has been used commonly as an index for studying electrotonic coupling (11,21,22,25, 36). Although gap junctions have not been reported in the IC, a gene encoding connexin (Cx36), a gap junction protein, which is synthesized by neurons only, has been found to be expressed in neurons of adult rat inferior colliculus (9,28).

In this study, it was aimed to study the possible presence of electrotonic coupling in the inferior colliculus of young rats using dye coupling as an index for gap junction.

Materials and Methods

Dissection of animal

For intracellular staining, slices from rat inferior colliculus were used. Seventy-six Wistar rats between 13 to 16 days of age were used for the experiments. Immediately after a rat was killed by cervical dislocation, the head was immersed in cold (4-8°C) oxygenated Na-free artificial cerebrospinal fluid (S-aCSF) (sodium

ions were replaced by sucrose on an equimolar basis) (2). Having removed the scalp and other soft tissues, the dorsal cranium was cut in order to expose the brain. The whole brain was rapidly removed and placed in freshly oxygenated cold S-aCSF. The brain stem containing the inferior colliculus and superior colliculus was freed from the rest of the brain by cutting transversally using a pair of scissors. Another transverse cut was made in the remaining block between the inferior colliculus and the cerebellum and the block caudal to the IC was discarded. After carefully removing the fine arteries over the surface of the inferior colliculus, using a pair of fine forceps, the specimen was mounted with a cyanoacrylate glue, with the superior colliculus end down, onto a mounting block. This block was fixed onto a Vibratome tray, which was filled with cold continuously oxygenated S-aCSF (3-8°C). Slices (300 µm in thick) were then cut using a series 1000 Vibratome (Technical Products International Inc.). The slices were transferred into fresh continuously oxygenated S-aCSF. The time between the cervical dislocation and the completion of slicing was typically less than 7 min.

Electrophysiology

The slices were kept in S-aCSF for at least 1 hour to allow recovery from trauma. A slice was then transferred to the recording chamber. The slice was perfused at a rate of 6 7 ml/min with artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10. All chemicals were obtained from BDH and were of AnalaR grade. The pH of aCSF was 7.4 (between 7.35 and 7.45) subsequent to saturation with 95% O_2, 5% CO_2 at 35°C. The osmolarity of the aCSF was in the range between 308-315 mOsm. Slices were viewed through an Olympus microscope with a maximum magnification of 50X and illuminated from above using a fiber optic light source. For stable recordings, tip resistance of the most suitable microelectrodes ranged between 140 and 180 MOhm. But only those microelectrodes that had the ability to pass currents of ≥2 nA were used. The recording were performed using an Axoclamp-2A amplifier (Axon Instruments Inc., Burlinghame).

Biocytin injection and histology

Intracellular staining was made with micro-electrodes filled with 2% solution of biocytin in 2 M K-acetate, buffered to pH of 7.4 with acetic acid. At the end of each successful recording, biocytin was injected into the impaled cell by iontophoresis using depolarizing current pulses of 1 nA amplitude with a duty cycle of 200 ms for varying duration between 1-30 min. Intracellular staining was carried out if the cell had resting membrane potentials more negative than -48 mV. The position of the injected neuron was noted on a sketch of the slice. More than ten minutes after the final injection of biocytin, the slice was removed from the recording chamber and then fixed for at least 24 hours in 2% glutaraldehyde 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The slices were then left in 30% sucrose in sodium phosphate buffer (0.1 M, pH 7.4) for about 24 hours for cryoprotection and were then cut into 50-60 µm sections on a freezing microtome. The sections were incubated for 2-3 hours at room temperature or overnight at 4°C with avidin D-HRP (Vector Laboratories) 20 µl in 10 ml sodium phosphate buffer (0.1 M, pH 7.4) with 1% triton X-100. Sections were rinsed in sodium phosphate buffer (0.1 M, pH 7.4) and the HRP was then reacted using the DAB-nickel/cobalt intensification method (1). Sections were rinsed with sodium phosphate buffer (0.1 M, pH 7.4) and were then mounted onto gelatine-coated slides. After drying them in air, they were dehydrated, counter stained with neutral red and coverslipped.

Results

Twenty-seven out of 76 biocytin injections into neurons in both the cortex regions and the central nucleus of the IC resulted in staining of more than one cell, dye coupling (Figure 1). In some cases, both the dye-coupled cells were equally well stained so that fine structures such as dendritic spines and fine dendritic arborizations were obvious in the coupled cells, in others the neuron into which biocytin was injected could be better distinguished by its darker appearance resulting from better filling.

Three dimensional analysis of the coupled cells showed that the point of contact between the dye-coupled neurons appeared to vary and included dendro-dendritic, soma-somatic and dendro-somatic contacts and their dendritic arborisations overlapped extensively (Figure 1 and 2). The number of coupled cells resulting from each injection and the incidence of coupling was correlated with the duration of the biocytin injections; the longer injections gave rise to more coupled cells and a higher incidence of coupling (Table 1). A biocytin injection of one minute duration, the typical duration for filling a cell, resulted in only two coupled cells at a 16.1% incidence, whereas an injection time of 5 to 10 minute gave rise to up to four coupled cells at a 28% incidence

Figure 3 shows the physiological behavior of the coupled neurons. There was no correlation between coupling and different firing patterns; the dye couplings were seen in neurons with onset, regular (Figure 3A) and

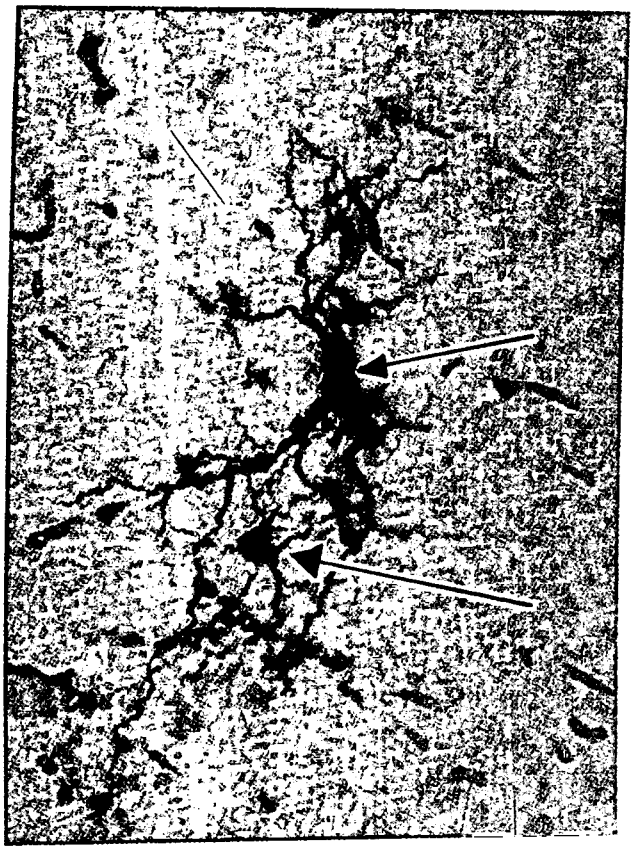


Figure 1. A photomicrograph of coupled neurons stained with intracellular biocytin in the inferior colliculus. Intracellular iontophoritic injection of biocytin into a single neuron resulted in staining of more than one cell. Two dye coupled neurons are indicated with arrows. Calibration: 33 µm.

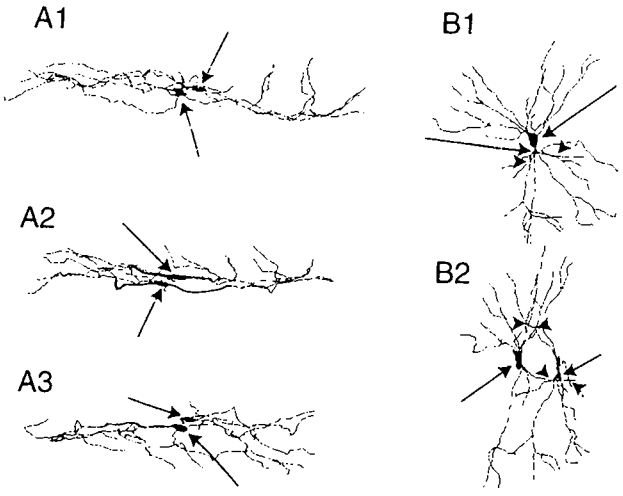
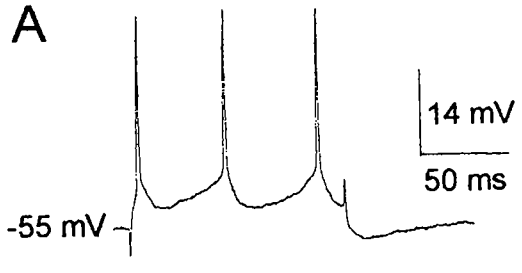


Figure 2. Three dimensional analysis of the coupled cells. A: There are two cells stained with biocytin injection. Three views of the coupled cells taken from different rotations angles were shown. B: Another coupled cells were shown at two different rotations. The somas of the cells were indicated by the arrows. There are numerous point of possible contacts between two cells, which are shown by the arrowheads.



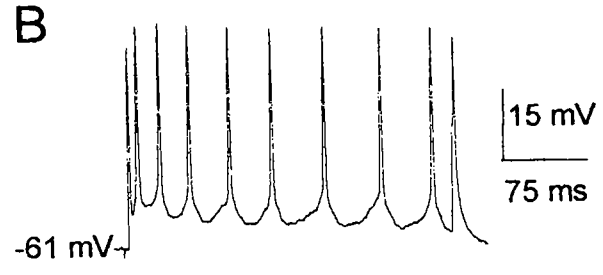


Figure 3. Electrophysiological responses recorded from two coupled cells. A: In response to 0.2 nA current, the cell fired action potential regularly. B: Another coupled cell responded with an adapting firing pattern in response to +0.2 nA current.

adapting (Figure 3B) temporal firing patterns. In order to test the possibility that this correlation might be the result of keeping the slices longer in the bath after injection of biocytin before fixation and was not the result of the longer biocytin injection, the injected slices were kept in the bath for two different time periods. As shown in Table 2, no correlation was found between long and short periods of incubation and with the pattern and incidence of the coupling.

The dye coupling was found to be correlated with the age of animal. The incidence rate of the coupling among stained cells was 28% in IC slice preparations taken from 13 day-old rats, whereas the rate was 15% in the 16 day-old group (correlation coefficient = -0.91). In order to prevent artefactual coupling, the following precautions were taken: only one neuron was injected with biocytin in each colliculus or the stained cells were at least 1 mm apart in a colliculus and penetrations were terminated if the action potential amplitude fell below 50% of the action potential's initial amplitude or if the cell stopped firing. However, in five cells, it was clear from the recording that the microelectrode had come out of the cell during the biocytin injection. In order to test whether neighboring cells picked up the dye from the extracellular space, the injection was carried out for the standard duration and then it was noted that no other cell was stained. In another six control experiments biocytin was injected into the extracellular space through microelectrodes of similar tip resistance for 10 min in two slices with the same iontophoretic currents. No intracellular uptake of the dye was observed as a result of

Table 1. The effect of duration of biocytin injection on number and pattern of dye coupling.

Duration of biocytin injection	Total number of staining	Total number of couplings	Number with 2 coupled cells	Number with 3-4 coupled cells	Incidence rate of coupling
5-10 min	25	7	4	3	28%
3 min	22	6	6	-	26%
1 min	29	5	5	-	16%

Table 2. The effect of slice incubation duration after biocytin injection on the number and pattern of dye coupling.

Period of incubation in bath after starting injection prior to fixation	Total number of stainings	Total number of couplings	Number with 2 coupled cell	Number with 3-4 coupled cells	Incidence rate of coupling
20-30 min	51	12	10	2	23.5%
50-120 min	25	6	5	1	24%

this procedure and conversely no extracellular dye was observed after single cell injections when the precautions given above were followed.

Discussion and Conclusion

The results reported here of dye–coupling among inferior colliculus cells supports the existence of electrical coupling among these cells though direct demonstration of electrotonic coupling is absent. On the basis of the control experiments and the precautions taken, it is unlikely that biocytin leaked out of the cells and was subsequently taken up by neighboring neurons or that there was inadvertent staining due to possible leakage during previous brief cell penetrations while searching for a stable cell. Furthermore, the observation that some of the dye-coupled cells were more than 0.1 mm apart rules out this possibility, because there were a number of unstained cells between these stained neurons.

On the other hand, it could be argued in the dye-coupled cases that dye crossed chemical synapses. This argument has, in fact, been suggested to explain the dye coupling occurring as a result of injection of Procion yellow into single neurons but not Lucifer yellow or biocytin revealed coupling (19). It is reported that biocytin does not cross chemical synapses (18). The observation that the pattern and incidence of dye coupling was not found to be correlated with the incubation duration of the slices between biocytin injection and fixation could be regarded as evidence against this possibility. Therefore, on the basis of these data presented here and with the light of the evidence that the gap junction gene (Cx36) has been expressed in adult rat inferior colliculus (9,28). It is suggested that gap junctions probably exist in the rat inferior colliculus, in the same way that existence has been suggested on an ultrastructural basis in cochlear nucleus (26,32,33) and primary auditory cortex (30,31). Gap junctions may be an important physiological mechanism in the auditory pathway since conduction between cells through gap junctions is faster than that through chemical synapsis. The functioning of vertebrate hearing is crucially dependent on the ability of neurons to encode timing. Human can distinguish 2° differences in the location of sound source in the horizontal plane, which indicates that they can detect differences of 11 microseconds (23). This ability may be dependent on gap junctional conductance.

It has been shown that gap junctional conductance is not a static mechanism but a dynamic one controlled by several factors, including neurotransmitters, pH, voltage, adenosine 3, 5-cyclic monophosphate etc. (16,28). For example, dopamine and histamine, neurotransmitters, which are considered to have modulatory actions on chemical transmission in many structures of the mammalian brain, have been shown to enhance electrotonic coupling via gap junctions (15,24,27). Although it is not known whether dopamine functions as a neurotransmitter in the IC of rat, there are reports that growing numbers of neurotransmitters have similar modulatory effects, as reviewed by Jefferys (16) and Perez Velazquez and Carlen (28).

However, it is pure speculation to attribute any function to the gap junctions at this stage. There are growing evidence which suggests that gap junctions in mature mammalian nerveous system may be a residue left from their role in embryonic development; they may be present in a dormant state waiting to be activated in abnormal brain rhythms (8,13,14,17,35). They might have also been reactivated from a dormant development

mechanism by the physical damage to the neurons during slicing, with dendrotomy (7,14). Electrotonic coupling is widespread throughout the developing nervous system but they are thought to be transient, disappearing at the end of first few postnatal weeks in most types of neurons (4,8,17,29). Their function in the early development may be the transport of messenger molecules between neurons, pattern formation, neurogenesis, neuronal differentiation and circuit formation (8,10,28,35,37).

It is concluded that the gap junctions revealed by dye coupling in IC preparation of 13-16 day-old rats are more likely to be a residue left from their role in embryonic development. There are two pieces of evidence for this statement. First, the incidence rate of the coupling among stained cells was higher in 13 day-old rats than that in the 16 day-old ones; this age may coincide with the transition to normal chemical communication. Second, expression of the gene for Cx36 in adult rat IC is low compared to some other brain areas in which gap junctions are proved to exist.

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