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THE LOCALIZATION AND PHYSIOLOGICAL EFFECTS OF CANNABINOID RECEPTOR 1 IN THE BRAIN STEM AUDITORY SYSTEM OF THE CHICK

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Abstract—Fast, temporally-precise, and consistent synaptic transmission is required to encode features of acoustic stimuli. Neurons of nucleus magnocellularis (NM) in the auditory brain stem of the chick possess numerous adaptations to optimize the coding of temporal information. One potential problem for the system is the depression of synaptic transmission during a prolonged stimulus. The present study tested the hypothesis that cannabinoid receptor 1 (CB1) signaling may limit synaptic depression at the auditory nerve-NM synapse. *In situ* hybridization was used to confirm that CB1 mRNA is expressed in the cochlear ganglion; immunohistochemistry was used to confirm the presence of CB1 protein in NM. These findings are consistent with the common presynaptic locus of CB1 in the brain. Rate-dependent synaptic depression was then examined in a brain slice preparation before and after administration of WIN 55,212–2 (WIN), a potent CB1 agonist. WIN decreased the amplitude of excitatory postsynaptic currents (EPSCs) and also reduced depression across a train of stimuli. The effect was most obvious late in the pulse train and during high rates of stimulation. This CB1-mediated influence could allow for lower, but more consistent activation of NM neurons, which could be of importance for optimizing the coding of prolonged, temporally-locked acoustic stimuli.

Key words: cochlear nucleus, synaptic depression, nucleus magnocellularis, brain slice, WIN 55;212–2, calyx.

Acoustic stimuli drive high rates of neural activity and these stimuli can be relatively long-lasting. *In vivo* spontaneous firing rates average 100 Hz even lacking auditory stimuli (Warchol and Dallos, 1990). Neurons must be able to respond in a consistent manner throughout the entirety of the stimulus to code this information effectively. Additionally, the encoding of important information about acoustic stimuli is not simply a duration code as it requires that action potentials (APs) should be precisely timed with respect to the acoustic stimulus. Neurons in the brain stem auditory system possess several features that optimize their ability to maintain precisely timed and sustained responses.

Important adaptations to the rigorous demands of coding acoustic information have been identified in the cochlear nucleus of the chick, nucleus magnocellularis (NM). This area is key for processing time-locked signals from the auditory nerve (vestibulocochlear nerve [cnVIII]) and relaying that information to nucleus laminaris (NL), where the analyses of interaural time differences assist in tasks such as the localization of a sound source (Sullivan and Konishi, 1984; Takahashi et al., 1984; Carr and Konishi, 1990; Warchol and Dallos, 1990; Overholt et al., 1992).

One adaptation toward accomplishing NM's key role of accurately coding timed information can be seen in the nature of its innervation. NM neurons receive large excitatory calyceal input from only 2–3 cnVIII fibers (Carr and Boudreau, 1991). Each of these afferents is capable of producing suprathreshold currents through a large release of glutamate, resulting in a high neurotransmission safety factor (Parks and Rubel, 1978; Hackett et al., 1982; Zhang and Trussell, 1994a,b). NM neurons produce a single, strong excitatory postsynaptic potential (EPSP) mediated primarily through AMPA receptor activation. Little jitter or temporal summation is observed which is aided by low-threshold K^+ channels to help ensure a minimal refractory period (Reyes et al., 1994; Zhang and Trussell, 1994b).

While these synaptic adaptations allow NM to fire at very high rates, synaptic depression can become a serious impediment to the coding of ongoing auditory stimuli. This short-term effect is seen as a progressive decline in the size of postsynaptic responses to the same stimulus. AMPA receptor desensitization appears to be a major limitation of sustained responses (Trussell et al., 1993; Otis et al., 1996), but vesicle depletion presents a second limitation to sustained AP firing (Brenowitz and Trussell, 2001). In addition, glutamatatergic autoreceptor activation may contribute to this phenomenon (Barnes-Davies and Forsythe, 1995; von Gersdorff et al., 1997). Failing to limit synaptic depression would be detrimental to temporal coding as it would prevent NM from coding throughout the duration of a stimulus.

Strong synaptic depression is seen in both NM (Hackett et al., 1982; Zhang and Trussell, 1994a) and NL (Kuba et al., 2002; Cook et al., 2003) in brainstem slice prepara-

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Abbreviations: ABC, avidin-biotin-complex; ACSF, artificial cerebral spinal fluid; ANOVA, analysis of variance; AP, action potential; BSA, bovine serum albumin; CB, cannabinoid; CB1, cannabinoid receptor 1; CG, cochlear ganglion; cnVIII, vestibulocochlear nerve; DAB, diaminobenzidine; DMSO, dimethyl sulfoxide; E(n), embryonic day (n); eCB, endocannabinoid; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IHC, immunohistochemistry; ISH, *in situ* hybridization; MNTB, medial nucleus of the trapezoid body; NGS, normal goat serum; NHS, normal horse serum; NL, nucleus laminaris; NM, nucleus magnocellularis; P(n), posthatch day (n); PBS, phosphate buffered saline; SON, superior olivary nucleus; SSC, standard sodium citrate; WIN, WIN 55,212–2.

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tions. It is possible that the procedure itself, that is, extraction of a brain slice, removes endogenous signals necessary to attenuate depression. For example, descending GABAergic inhibition from the superior olivary nucleus (SON) may play a dual role, working both pre- and postsynaptically at the cnVIII-NM synapse to preserve temporal coding (Carr et al., 1989; Code et al., 1989; Lachica et al., 1994). $GABA_A$ receptors influence NM excitability, tightening the window for summation postsynaptically (Hyson et al., 1995; Monsivais et al., 2000), whereas $GABA_B$ receptors help conserve glutamate release during high rates of activity (Brenowitz et al., 1998). In doing so, $GABA_B$ activation reduces synaptic depression across a train of stimuli, limiting the progressive decrease in response.

Another potential mechanism for regulating glutamatergic signaling is cannabinoid (CB) signaling, which has already been shown to have a modulatory role in other sensory systems (Straiker et al., 1999a,b; Czesnik et al., 2007; Deshmukh et al., 2007; Thornton-Jones et al., 2007; Nyilas et al., 2009). Our laboratory recently reported the distribution of the cannabinoid receptor 1 (CB1) mRNA throughout the brain of the chick (Stincic and Hyson, 2008). While little mRNA expression was found in NM or NL, the cnVIII ganglion (vestibular portion) displayed robust CB1 mRNA expression. This finding suggested that CB1 mRNA expression may be found in the cochlear ganglion (CG) as well. CB1 is expressed in the dorsal cochlear nucleus of rats (Mailleux and Vanderhaeghen, 1992) and CBs can modulate synaptic plasticity in auditory nuclei (Penzo and Peña, 2009; Zhao et al., 2009). CB mediated retroinhibition has also been reported in the medial nucleus of the trapezoid body (MNTB) (Kushmerick et al., 2004). This is significant because the MNTB is an auditory nucleus that also receives calyceal input. Taken together, these previous reports suggest that CB signaling in the chick auditory brain stem is likely as well.

CB signaling acts primarily through metabotropic receptors that share some traits with classical neurotransmitter systems, but differs in key aspects. When activated, the receptors trigger second messenger signaling cascades through $G_{i/o}$-proteins (Howlett and Fleming, 1984; Howlett, 1985; Howlett et al., 1986). Vesicles, however, are not required for transport because endogenous CBs (eCBs) are lipophillic and capable of crossing the cell membrane either unaided or by facilitated diffusion (Beltramo et al., 1997; Hillard et al., 1997). Once released into the synapse from postsynaptic cells, eCBs activate CB receptors located on the presynaptic cell. Functional CB1 has been localized presynaptically to axonal terminals with almost no exceptions in adult animals (Schlicker and Kathmann, 2001).

A major effect of CB1 activation is inhibition of voltage-gated Ca^{2+} channels (N-type and P/Q-type) (Caulfield and Brown, 1992; Felder et al., 1993; Mackie et al., 1993), leading to a reduction in the number of vesicles released on arrival of the AP to the terminal. In this way, CB1 activation can reduce the amount of neurotransmitter released into the synaptic cleft, subsequently lessening the factors that contribute to synaptic depression.

If CB signaling is an additional adaptation for optimizing coding at the cnVIII-NM synapse, it must be present and effective at modulating synaptic transmission. Our study first establishes the presence of CB1 at the cnVIII-NM synapse using *in situ* hybridization (ISH) and immunohistochemistry (IHC), and then documents the influence of CB1 activation on synaptic depression.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were prehatch (E18-19) or posthatch (P10) chicks (*Gallus domesticus*) incubated at Florida State University from eggs obtained from a local supplier. Subjects of either sex were chosen randomly.

In situ hybridization

The 702 bp fragment of chick CB1 mRNA was re-subcloned into the pGEM vector (Promega, Madison, WI, USA). Both an antisense and a corresponding sense-control riboprobe were synthesized using a protocol adapted from previously published work (McCullumsmith et al., 2003; Krause et al., 2006). Alternating tissue sections were hybridized with either sense or antisense ^{35}S riboprobe and exposed to film.

For riboprobe synthesis, 8 μl of uridine 5''-(a-thio)triphosphate [35S] (PerkinElmer, NEG039H, Waltham, MA, USA) and 4.0 μl 5× transcription buffer, 2.0 μl of 0.1 mM dithiothreitol, 1.5 μl adenosine triphosphate, cytosine triphosphate and guanidine triphosphate, 2.0 μl linearized plasmid (1 $\mu g/mL^{-1}$) DNA 1.0 μl RNAse inhibitor (40 U μL^{-1}), and 1.5 μl T7 RNA polymerase (15 U μL^{-1}) was combined and incubated for 2 h at 37 °C. DNase (1.0 μl: RNase-free) was added and the mixture was incubated for 15 min at room temperature. Radio-labeled probe was purified with microspin chromatography columns (Bio-Rad, Hercules, CA, USA).

Slides were removed from −80 °C storage and postfixed in 4% paraformaldehyde at room temperature for 1 h. After fixation, the slides were washed in 2× standard sodium citrate, (SSC, where 1× SSC is 300 mM NaCl and 30 mM sodium citrate), pH 7.2, three times for 5 min each. Slides were then washed in deionized water for 1 min and placed in 0.1 mM triethanolamine (pH 8.0)-acetic anhydride, 400: 1 (v/v) on a stir plate, for 10 min. The final rinse was in 2× SSC for 5 min, followed by dehydration through graded alcohols and air-drying for 30 min. A coverslip with 60 μl of hybridization buffer (75% formamide,10% dextran sulfate, 3× SSC (pH 7.2), 50 mM Na_2HPO_4 (pH 7.4), 10 mM dithiothreitol, 1× Denhardt's solution (Sigma-Aldrich, St. Louis, MO, USA), 100 micrograms $(mL)^{-1}$ yeast tRNA) was placed on each slide. Strength of probe in 60 μl of hybridization buffer was approximately 1–2 million counts per minute as assessed by a liquid scintillation counter (Beckman Coulter, Brea, CA, USA). Slides were placed in a covered tray with filter paper saturated with 75% formamide. After overnight incubation at 55 °C, coverslips were removed and slides were placed at room temperature in 2× SSC for 5 min, followed by RNase (200 micrograms $[mL]^{-1}$ in 10 mM Tris–HCl, pH 8.0, 0.5 mM NaCl) at 37 °C for 30 min and then: 2× SSC at room temperature for 10 min, 1× SSC for 10 min at room temperature; 0.5× SSC at 55 °C for 60 min; and 0.5× SSC for 10 min at room temperature. The slides were dehydrated in graded ethanol solutions, air dried, placed in X-ray cassettes, and apposed to BioMax MR Film for 10 days.