REVIEW

Purinergic Modulation of Activity in the Developing Auditory Pathway

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Abstract Purinergic P2 receptors, activated by endogenous ATP, are prominently expressed on neuronal and non-neuronal cells during development of the auditory periphery and central auditory neurons. In the mature cochlea, extracellular ATP contributes to ion homeostasis, and has a protective function against noise exposure. Here, we focus on the modulation of activity by extracellular ATP during early postnatal development of the lower auditory pathway. In mammals, spontaneous patterned activity is conveyed along afferent auditory pathways before the onset of acoustically evoked signal processing. During this critical developmental period, inner hair cells fire bursts of action potentials that are believed to provide a developmental code for synaptic maturation and refinement of auditory circuits, thereby establishing a precise tonotopic organization. Endogenous ATP-release triggers such patterned activity by raising the extracellular K^+ concentration and contributes to firing by increasing the excitability of auditory nerve fibers, spiral ganglion neurons, and specific neuron types within the auditory brainstem, through the activation of diverse P2 receptors. We review recent studies that provide new models on the contribution of purinergic signaling to early development of the afferent auditory pathway. Further, we discuss potential future directions of purinergic research in the auditory system.

Keywords Purinergic signaling · Auditory system · Development · Cochlea · Spiral ganglion · Auditory brainstem

Introduction

In the early 1970s ATP was recognized as a neurotransmitter molecule [1], opening a whole new research field of purinergic transmission and cellular signaling. Numerous studies revealed the importance of purinergic signaling in glial and neuronal communication, under both physiological and pathological conditions [2]. It was postulated that ATP is stored at different concentrations in probably every neuronal synaptic vesicle [3]. Vesicular release of ATP, and its co-release with other neurotransmitters, occur from both neurons and astrocytes [4–11]. Alternatively, ATP can be delivered to the extracellular space via membrane channels, such as connexin hemichannels [12–14], pannexin 1 [15–17], Ca^{2+} homeostasis modulator 1–3 [18–20], volume-regulated anion channels [21, 22], maxi-anion channels [23], and the P2X7 receptor [24]. Once released, extracellular ATP and its breakdown product adenosine exert a wide range of cellular effects by activating plasma membrane-localized receptors.

Purinoreceptors were first described in 1976 [25], and thereafter classified into two receptor families: P1 and P2, based on their affinity for adenosine and ATP/ADP [26]. Over the following years, an expanded nomenclature has been established: P2 receptors were further divided in two families, P2X and P2Y, each containing several subtypes, while P1 receptors were denominated as adenosine receptors [27]. P2X receptors are trimers or hexamers formed from individual subunits encoded by seven distinct genes and designated P2X1 to P2X7 [28]. They assemble to form ATP-gated ion channels, permeable to Na^+ , K^+ , and Ca^{2+} [29, 30]. All P2Y receptors are coupled to G-proteins, typically composed of seven transmembrane domains. In mammals, there are eight P2Y receptors divided into two subgroups based on the G-protein engagement: P2Y1,

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P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors are coupled to G_q and activate the phospholipase C-inositol 1,4,5-triphosphate pathway, while the receptors of the second subgroup, composed of P2Y₁₂, P2Y₁₃, and P2Y₁₄, couple to G_{i/o} and inhibit adenylyl cyclase [31]. Four adenosine receptors have been cloned and characterized so far: A₁, A_{2A}, A_{2B}, and A₃. A₁ and A₃ are coupled to the G_{i/o} that reduces cAMP levels via inhibition of adenylyl cyclase. On the other hand, A_{2A} and A_{2B} activate G_s proteins and stimulate cAMP production [32].

Purinergic signaling is an essential component of sensory transduction and information coding in the visual, auditory, olfactory, and gustatory systems [33–35]. Along the ascending auditory pathway, purinergic receptors are expressed in many cell types (cochlea and spiral ganglion neurons (SGNs) [35]; cochlear nucleus (CN) [36]; medial nucleus of the trapezoid body (MNTB) [37]; and lateral and medial superior olive (LSO and MSO) [38]). In past years, we have gained new insights into the role and mechanisms of purinergic signaling in the developing auditory system, including propagation of Ca²⁺ waves across the inner ear [39, 40], modulation of Ca²⁺ spikes in inner hair cells (IHCs) [41–43], refinement and stimulation of outer hair cell (OHC) afferents [44, 45], and modulation of transmitter release and action potential (AP) discharges at specific brainstem synapses [46–48].

The extraction of sound frequencies from complex sound stimuli is accomplished in the inner ear, through the excitation of hair cells. Based on their position and the specific physical properties of that particular portion of the basilar membrane, the hair cells are excited only by a narrow band of sound frequencies [49]. This so-called tonotopic organization gives rise to precise maps of frequencies in all nuclei of the ascending auditory pathway up to the auditory cortex [50]. Similar to topographically arranged sensory maps in the visual and somatosensory systems [51–53], the establishment of tonotopic maps depends on two successive processes: (1) the genetically programmed distribution of molecular cues guiding the appropriate axonal projections to their synaptic partners, and (2) the activity-dependent refinement of such established connections [54–56]. In the auditory system, rearrangement of primary synaptic contacts begins before hearing onset [57, 58], guided by the spontaneous patterned activity generated in the inner ear and conveyed from there along the afferent auditory pathway [41, 59–62]. To what extent and through which mechanisms purinergic signaling contributes to the bursting activity of hair cells and, subsequently, central auditory neurons, is still a matter of debate. This review focuses on recent findings on the role of ATP in the cochlea and auditory brainstem during the sensitive developmental phase of the auditory system before and shortly after hearing onset.

Purinergic Signaling in the Developing Cochlea

The cochlea is a spiral-shaped cavity within the bony labyrinth of the inner ear containing the organ of Corti, the sensory epithelium made up of the two types of sensory hair cells, embedded in several types of supporting cells (Fig. 1). The IHCs serve as transducers of a sound-evoked mechanical stimulus into a graded receptor potential, while the OHCs contribute to the increase in frequency selectivity and hearing sensitivity as part of an active nonlinear amplifier [63, 64]. The adult-like cytoarchitecture and function of the organ of Corti is fully established shortly after the onset of acoustically evoked signal processing (subsequently termed ‘hearing onset’ for the sake of brevity) [65, 66], which in altricial rodents is around postnatal day twelve (P12) (mouse [61, 67–69]; gerbil [70, 71]; rat [72]). Several physiological and anatomical features characterize an immature cochlea: (1) spontaneous Ca²⁺ waves which spread throughout the organ of Corti, at that time organized into the greater epithelial ridge containing Kölliker’s organ, IHCs, inner phalangeal cells, and border cells, and the lesser epithelial ridge, comprising OHCs and lateral non-sensory supporting cells [73, 74]; (2) Ca²⁺-dependent regenerative APs generated by IHCs and OHCs [41, 75]; and (3) reorganization of the afferent fibers of the SGNs which initially innervated both IHCs and OHCs, and subsequently establish the morphologically distinct IHC-type I SGN-fiber synapses and OHC-type II SGN-fiber synapses [76, 77].

Intercellular Ca²⁺ waves, spontaneous Ca²⁺ APs, and refinement of hair cell-SGN synapses are essential for the development of the Organ of Corti, as well as for the structural reorganization of neuronal circuitries in the central auditory system [44, 58, 75, 76, 78–85]. There is ample evidence that purinergic signaling plays an important role in these early developmental processes. The current hypotheses are discussed below.

Inner Hair Cells

In adult animals, processing of acoustic information starts with transduction of mechanical pressure oscillations (sound wave) into graded receptor potentials of IHCs and electrical AP firing of SGNs. Before hearing onset, IHCs are irresponsive to sound, in part because of the closed external auditory meatus. Still, IHCs exhibit spontaneous Ca²⁺-dependent spikes, evoking bursts of APs in type I afferent fibers of SGNs, as shown by numerous studies in recent years crucially contributing to our understanding of the functional development of the cochlea.

IHCs in the immature cochlea generate Ca²⁺ APs, whereas mature IHCs encode sound amplitude by graded

Fig. 1 Schematic of the organ of Corti as in prehearing mice, shown in cross section. Two types of sensory cells - inner hair cells (IHCs) and outer hair cells (OHCs) are tightly packed between the immature tectorial membrane (TM) and several types of supporting cells that control the homeostasis of extracellular fluids. P2 receptors are differentially expressed in sensory and supporting cells. The inset

depicts the position of the organ of Corti within the temporal bone relative to other middle and inner ear structures. KO, Kölliker's organ; ISC, inner supporting cell; OSC, outer supporting cell; Type I and II AF, spiral ganglion neuron afferent fibers; PC, pillar cell; BM, basilar membrane; DC, Deiters' cell.

receptor potentials [80, 86–88]. This transition, occurring around hearing onset, is mediated by the developmental change in the pattern of K^+ channel expression in the IHC [89, 90]. Beforehand, i.e. during the first two postnatal weeks, ATP-mediated currents occur spontaneously in Kölliker's organ [42]. This transient structure located medial to IHCs (Fig. 1) consists of tall columnar inner supporting cells (ISCs) which slowly degrade after hearing onset [73, 91]. Via ATP, the ISCs elicit the spreading intercellular Ca^{2+} waves that reach IHCs and affect their activity [41, 92]. The exact mechanism of initiation and expansion of these Ca^{2+} signals is still unclear, but they bear similarities with Ca^{2+} waves found in glia, neurons, epithelial cells, and many other cell types [93]. In the organ of Corti, intercellular Ca^{2+} waves seem to be initiated by the release of ATP via connexin Cx26 and Cx30 hemichannels [39, 94, 95], followed by the autocrine and paracrine activation of P2Y1, P2Y2, and P2Y4 receptors, and successive triggering of the PLC-IP₃ pathway (Fig. 2) [96, 97]. Hemichannels are also able to release IP₃ into the extracellular space, where it could bind to IP₃ receptors expressed on the surface of supporting cells and potentiate the spread of Ca^{2+} waves [98, 99]. Additionally, connexin Cx26 and Cx30 build homo- and heteromeric gap junction proteins that allow the passage of IP₃ and Ca^{2+} between cells (Fig. 2), thus enabling further propagation of intercellular Ca^{2+} waves [39, 92, 100]. Intracellular Ca^{2+} transients open Ca^{2+} -activated Cl^- channels TMEM16A, thus allowing the efflux of Cl^- ions, followed by charge-

balancing K^+ efflux and water osmosis, resulting in crenation, i.e. shrinking of ISCs [101]. When Ca^{2+} waves reach the vicinity of IHCs, elevated levels of extracellular K^+ initiate the following sequence of events: depolarization of the surrounding IHCs, generation of Ca^{2+} spikes, and glutamate release from IHCs, which finally triggers AP bursts in SGNs [101]. The accompanying crenation of ISCs enhances K^+ clearance, thereby limiting the exposure of IHC to elevated $[K^+]_e$ and preventing tonic IHC firing. This mechanism is thought to sculpt and coordinate firing activity in small groups of neighboring IHCs and SGNs [96, 101, 102]. To what extent ATP-induced Ca^{2+} waves influence the firing of IHCs, and subsequently SGN afferent fibers, is still a matter of debate. Yet, it is important to consider the different properties of immature IHCs: they fire spontaneous Ca^{2+} APs during the first postnatal week due to their depolarized resting membrane potential [43, 103], whereas in the second week, an external trigger in the form of ATP or an ATP-induced increase in $[K^+]_e$ is required to elicit firing [42, 101]. This is in agreement with the finding that P4-6 IHCs generate single, or several successive, fast Ca^{2+} transients, independent of Ca^{2+} waves originating in Kölliker's organ [40]. Still, Ca^{2+} waves in an ISC trigger additional bursts of Ca^{2+} transients in several neighboring IHCs, thereby enhancing and synchronizing their activity. Consistent with intrinsic AP firing in early postnatal IHCs, their activity is normal in Cx26 and Cx30 KO mice, which are devoid of Ca^{2+} waves in ISCs [84]. However, these IHCs fail to

Fig. 2 Schematic of the proposed mechanism controlling the inner hair cell membrane potential. ATP, spontaneously released through connexin Cx 26 and Cx 30 hemichannels (1), binds to the auto- and heteroreceptors P2Y1, P2Y2, and P2Y4 (2) on inner supporting cells (ISCs). G-protein coupled P2YRs activate the PLC-IP₃ signaling pathway that elevates the intracellular Ca²⁺ concentration. Connexin gap junctions (3) allow diffusion of IP₃ and Ca²⁺ to neighboring ISCs, thus building an intercellular Ca²⁺ wave that reaches the vicinity of IHCs. Elevated [Ca²⁺]_i activates the TMEM16A channel (4), leading to Cl⁻ efflux that is balanced by K⁺ efflux through the leak K⁺ channels (5). The rise in extracellular K⁺ concentration depolarizes the IHC, resulting in a series of Ca²⁺-dependent APs shaped mainly by the K_V delayed rectifier K⁺ channel (6) and the Ca_V1.3 Ca²⁺ channel (7). Further sculpting of the IHC firing pattern is achieved via SK2 K⁺ channel (8) that is activated by the Ca²⁺ influx through the surrounding α9α10 nicotinic ACh receptors (9) and P2X3 receptors (10). Additional Ca²⁺ entry and IHC depolarization might be achieved through P2X2 receptors (11) located in the proximity of stereocilia. AF, spiral ganglion neuron afferent fiber; EF, medial olivocochlear efferent fiber.

develop adult-like properties, suggesting a role of Ca²⁺ waves in shaping bursting activity during the prehearing period and thereby promoting maturation [83, 104].

Another intriguing question is whether ATP can directly instigate/modulate the activity of immature IHCs. Several lines of evidence imply that ATP can affect IHCs through P2XRs: (1) IHCs express P2X2, P2X3, and P2X7 receptors [105–108]; (2) rat IHCs show ATP-induced inward currents starting as early as P1, with the largest amplitudes shortly before hearing onset [42]; and (3) focal application of 10 μmol/L–100 μmol/L ATP on neonatal IHCs elicits trains of APs in postsynaptic SGNs [41, 62]. Furthermore, superfusion of the cochlea with 100 μmol/L ATP also depolarizes IHCs in mouse and gerbil [43, 103]. However, application of the P2XR antagonist TNP-ATP causes IHC

depolarization as well, while superfusion of low concentrations of ATP (3 nmol/L–10 nmol/L) hyperpolarizes IHCs [43]. These seemingly paradoxical results are explained by the putative involvement of different P2Rs (Fig. 2): (1) P2X2Rs in the proximity of stereocilia, activated by higher ATP concentrations might cause the IHC depolarization, and (2) P2X3Rs, coupled to SK2 channels at the IHC base and activated by nanomolar ATP concentrations might, on the contrary, lead to the hyperpolarization (Fig. 2) [43]. It is worth noting that SK2 channels are also indirectly coupled to heteromeric α9α10 nicotinic acetylcholine (ACh) receptors (Fig. 2). Their activation by efferent olivocochlear fibers causes Ca²⁺ influx, which in turn leads to opening of SK2 channels, hyperpolarization of IHCs through K⁺-efflux, and subsequent modulation of the AP discharge pattern [43, 103, 109–112]. Hence, ATP might act synergistically with ACh by modulating SK2 currents [43]. The studies showing that ATP application on IHCs elicits inward currents and depolarization of IHCs and AP firing in SGNs [41–43, 62, 103] were published before the mechanism of ISC-[K⁺]_e-elicited IHC-activation had been revealed. Therefore, a potential indirect activation of IHCs via ISCs cannot be excluded, because the experimental setting in these earlier studies included the latter cells. On the other hand, in a recent study of the neonatal mouse cochlea, IHCs were probed by applying 1 μmol/L ATP from the pillar side to avoid an indirect effect through the ISCs [40]. Here, ATP application evoked bursts of Ca²⁺ transients in IHCs that preceded Ca²⁺ elevations in ISCs, consistent with direct stimulation of P2Rs on IHCs. However, the ATP concentration around active ISCs sufficient for further spreading of Ca²⁺ waves is estimated to be < 1 μmol/L [113], while the amount of endogenous ATP around immature IHCs, and whether it is sufficient for a functional effect, remains to be elucidated.

In summary, ATP instigates intercellular Ca²⁺ waves spreading through the developing cochlea, causing a K⁺-efflux from ISCs, and eventually depolarizing IHCs. In the first postnatal week, extracellular K⁺ synchronizes the intrinsic spiking of neighboring IHCs. Following the development of a hyperpolarized resting membrane potential during the second postnatal week, large Ca²⁺ waves arise as a necessary trigger of IHC bursting.

Outer Hair Cells

In mammals, locus-specific oscillation of OHCs along the basilar membrane amplifies the mechanical stimulus at corresponding IHCs in a tonotopic manner, increasing the sensitivity, frequency selectivity, and dynamic range of cochlear mechano-electrical stimulus transduction (“cochlear active process”) [63, 114–116]. The

electromotility of mature OHCs can be reduced by ATP, thus affecting hearing sensitivity [12, 117]. In juvenile mice, purinergic signaling has been linked to the function of type II SGN fibers [45]. OHCs are contacted by the efferent fibers of medial olivocochlear neurons and by afferent fibers originating in type II SGNs. The former innervation conveys a suppression of OHC activity, serving to aid the detection of signals in noise and protect the cochlea from high-level sounds [118]. The role of afferent type II fibers is, on the other hand, still inconclusive [119]. One possible function is to drive the medial olivocochlear reflex, thus creating a negative feedback control of OHCs [120]. Another possibility is a nociceptive function through their concomitant activation via glutamate and ATP at trauma-inducing sound levels [121, 122].

During the first three postnatal weeks, type II fibers exhibit small and slow glutamatergic excitatory postsynaptic currents (EPSCs) elicited by sporadic vesicular glutamate release from OHCs [45]. It has been proposed that only concerted activation of groups of OHCs by very loud stimuli elicits AP firing in type II fibers [123, 124]. Type II fibers might also be depolarized by ATP released from supporting cells, possibly in response to damage of surrounding OHCs [45, 122]. It is possible that ATP evokes two distinct responses in type II afferents: high concentrations most likely stimulate P2X2Rs [106, 122], while low, submicromolar ATP preferentially activates G-protein-coupled P2Y receptors, apparently P2Y2Rs, resulting in closure of voltage-dependent KCNQ channels and higher excitability of SGN fibers [122, 125].

The OHC–type II afferent fiber synapse is established early in development (mouse: between E18 and P0 [76]), undergoing a substantial refinement in the first postnatal week, when OHCs exhibit Ca^{2+} -dependent spikes similar to IHCs [75, 126]. The Ca^{2+} waves originating from Kölliker's organ spread to the lesser epithelial ridge and activate P2YRs on Deiters' cells, the non-sensory supporting cells underneath OHCs [44, 127]. Through the connexin hemichannels, Deiters' cells release ATP, activating autologous P2YRs, but also the P2X3Rs of OHCs. The latter synchronize the otherwise spontaneous and random OHC Ca^{2+} signals and increase their firing rate [44]. This process is necessary for the proper refinement of the afferent projections onto OHCs, and for the maturation of OHCs, especially those in the apical cochlear region [75]. Additionally, type II fibers can be directly modulated by ATP, even shortly after hearing onset, which may contribute to synaptic maturation [45]. It still remains to be investigated whether the spontaneous OHC activity contributes to the bursting pattern of type II SGNs. Nevertheless, it can be concluded that purinergic signaling plays an important role in the maturation of OHC synapses and in the activation of type II SGN fibers.

Spiral Ganglion Neurons

SGNs are a heterogeneous group of bipolar neurons sending their peripheral afferents to the organ of Corti, while their central axons compose the auditory nerve (AN) projecting to the CN [128–130]. Type I SGNs account for 90%–95% of the primary afferent fibers, innervating a single IHC in groups of 5–30 [131]. Myelinated axons of type I SGNs are perfectly suited for fast transmission of APs from the cochlea, representing the main input to the central auditory system. The remaining 5%–10% are extensively branched afferents of type II SGNs, synapsing on multiple OHCs. The thin unmyelinated axons of type II SGNs project to the granule-cell region of the CN, probably relaying nociceptive information about trauma-inducing sounds [121, 129]. At birth, IHCs and OHCs are innervated by both types of SGN afferents, after which extensive pruning of the fibers in the first postnatal week leads to the adult-like wiring of hair cells [76, 77]. During this period, distinct P2Rs are expressed on type I and II SGNs, playing an important role in the synaptic refinement.

Both types of SGNs exhibit comparable ATP-gated somatic currents [132]. Several P2XRs reach maximal expression in SGNs during the first (P2X1 [133]; P2X3 [107, 134]; P2X7 [108]) and the second postnatal week (P2X2 [135]). In addition, five types of P2YRs are expressed in SGNs before the onset of hearing (P2Y1, P2Y2, P2Y4, P2Y6 and P2Y12) [125]. A developmental role has been assigned to the P2X2/3 heteromeric receptor for promoting reorganization of promiscuous SGN innervation of hair cells [136]. This was shown by exposing P4 spiral ganglion explants to ATP analogues. Activation of P2X2/3 receptors inhibited BDNF-mediated neurite growth and branching, indicating an effect on pruning and the retraction of promiscuous SGN neurites from hair cells.

In addition to the role in refinement of hair cell–SGN synapses, purinergic signaling modulates the AP activity of SGNs. APs in type I SGNs are generally triggered by glutamate released from IHCs [62, 137, 138]. Extracellular ATP was shown to raise intracellular Ca^{2+} in the fiber and soma of type I SGNs [139], and puff application of ATP onto isolated type I SGNs evoked inward somatic currents [140]. Still, at SGN dendrites (IHC–SGN type I synapse), exogenous ATP induced only a small subthreshold voltage change in the presence of glutamate receptor blockers, failing to elicit APs [41]. Consistent with a rather modulatory role, the somatic P2YRs have been suggested to account for facilitation of AP propagation along type I nerve fibers [140, 141]. In contrast, the activation of type II SGN fibers seems to be mediated by both P2X and P2Y

receptors, which seem to be potent enough to trigger APs [45, 122].

In conclusion, current data suggest direct modulation of both IHCs and OHCs and the respective type I and type II SGNs through yet not fully revealed combinations of P2Rs. Also, IHCs and type I SGNs can be indirectly activated, through the instigation of Ca^{2+} -waves in Kölliker's organ, subsequently causing a rise in the extracellular K^+ concentration. Apparently, both mechanisms play crucial roles in the postnatal maturation of hair cells and SGNs, and in the refinement of their synaptic connections. Furthermore, by modulating spontaneous bursting activity of SGNs that is conveyed to the central nervous system, purinergic signaling may also contribute to the development of upstream brainstem nuclei.

Purinergic Signaling in the Auditory Brainstem

Sound features are encoded into series of APs, generated by SGNs (N. VIII pars cochlearis) and conveyed by their axons to second-order auditory brainstem neurons in the CN [142]. They in turn project to the third-order neurons, such as nuclei of the superior olivary complex (Fig. 3), nuclei of the lateral lemniscus, and to the inferior colliculus in the midbrain [143]. Distinct neuron types within the auditory brainstem express P2 receptors, and several studies have shown the effects of purinergic signaling on neuronal activity during early development.

Cochlear Nucleus

In the CN, each AN fiber bifurcates into an ascending branch projecting to the anteroventral cochlear nucleus (AVCN), and a descending branch projecting to the posteroventral (PVCN) and dorsal cochlear nuclei [142]. Thus, each AN fiber projects to all three tonotopically organized CN partitions [144]. There, different types of second-order neurons form the starting points of distinct ascending auditory processing pathways. Sound source localization relies on the integration of input signals generated at the two ears and conveyed from the ventral CN bushy cells (BCs) directly to the MSO and LSO, or indirectly via the MNTB (Fig. 3) [143, 145]. Large spherical BCs (see Fig. 3 SBC), densely packed in the anterior portion of the ventral CN, receive one to three large axosomatic terminals, i.e. the endbulbs of Held [146]. Small globular BCs (see Fig. 3 GBC) of the caudal AVCN and PVCN receive ~ 17 modified endbulbs [147]. Convergence of multiple excitatory inputs is thought to enhance the temporal accuracy of the BC firing with respect to presynaptic AN fibers [148]. Still, BCs do not function as a simple relay, since additional excitatory (cholinergic) and inhibitory (GABAergic/glycinergic) inputs shape their acoustically evoked firing [149–158]. In addition, recent *in vitro* and *in vivo* electrophysiological studies of BCs in gerbils provided evidence that ATP evokes membrane depolarization, and modulates the duration and frequency of APs during the early postnatal period

Fig. 3 Mammalian auditory brainstem with schematic wiring. Acoustic information is transduced into graded receptor potential by the inner hair cells (IHCs). This analog information is encoded into action potentials, conveyed by auditory nerve (AN) fibers. In the cochlear nucleus, the obligatory first central station along the afferent pathway, the information is segregated into streams processing different sound features. In the anteroventral part of the cochlear nucleus (AVCN), the major targets of AN fibers are stellate cells (multipolar cells, SCs), spherical bushy cells (SBCs), and globular

bushy cells (GBCs). The medial superior olive (MSO) is the first stage of processing of interaural time differences through the computation of bilateral excitatory inputs from the SBC plus an inhibitory projection from the medial nucleus of the trapezoid body (MNTB). Note that MNTB neurons are activated by the contralateral GBCs. Interaural level differences are first calculated in the lateral superior olive (LSO), where excitatory input from the ipsilateral SBC and inhibitory input from the ipsilateral MNTB converge. Excitatory neurons are shown in green and yellow, inhibitory neurons in red.

[36, 46, 159]. Previous investigations had documented the expression of P2X2 receptor in the adult rat CN [37, 160, 161], but a possible contribution of purinergic signaling to nuclear development remained unknown. Using a wide range of electrophysiological, pharmacological, and histological methods, it was shown that BC somata express heteromeric P2X2/3 and P2Y1 receptors [36, 46, 159]. The Ca^{2+} entry through P2X2/3 activates PKC and thereby increases AP duration and frequency of firing. These effects persist for seconds [159], and this ongoing modulation of the firing activity is most likely mediated through a Ca^{2+} -PKC-induced attenuation of the K_v conductance. Such modulation allows a larger Ca^{2+} influx [162] which might account for various forms of synaptic plasticity [163–165], and potentially also for the functional maturation of the endbulb of Held–BC synapse. Possible direct modulation of glutamate receptors through P2X2/3Rs can be excluded [159]. The activation of P2Y1Rs, on the other hand, is not sufficient to increase the AP firing *per se*, despite the induction of prominent Ca^{2+} release from internal stores [36].

The strongest ATP effects on BC activity occur before hearing onset, slowly waning until P23 [36, 46, 159]. This time-course matches the maturation profile of AMPA currents [159], and might be a compensatory mechanism to facilitate spiking during the period of pronounced AMPAR desensitization in immature BCs [166–168]. Direct application of ATP *in vivo* can evoke BC spiking under the pharmacological inhibition of glutamate receptors [46]. However endogenously released ATP does not seem to evoke APs independent of an AN drive, but rather facilitates the generation of glutamate-mediated APs by shortening the excitatory postsynaptic potential–AP delay [159]. In the first postnatal week, BCs are sensitive to ATP throughout the ventral CN. In the further course of development, the responsiveness to ATP becomes topographically restricted to low-frequency BCs in the rostral AVCN [159]. Stellate cells (SCs), the other principal neuronal type in the CN, are devoid of P2Rs at both neonatal and adult stages [36, 46, 159]. Considering the common innervation pattern, but different developmental time-courses of BCs and SCs [68], it is conceivable that purinergic signaling might contribute to the faster maturation of auditory processing in BCs.

The origin of ATP in the CN remains elusive. The possibility of a co-release of ATP with glutamate from excitatory endbulb terminals, or with GABA or glycine from inhibitory inputs can be excluded by stimulation of the respective inputs in acute slice preparations [46]. Therefore, ATP might originate from astrocytes, with glutamate from the endbulb triggering its release as part of a complex tripartite synapse [169]. Astrocyte-derived ATP

has been shown to promote the postsynaptic insertion of AMPA receptors, the phosphorylation-dependent inhibition of postsynaptic and extrasynaptic GABA receptors, and the Ca^{2+} -dependent inactivation of postsynaptic NMDA receptors, thus affecting the neuronal excitability and/or bidirectional modulation of synaptic strength [7, 170–173].

Taken together, endogenously released ATP specifically modulates the activity of BCs in a tonotopic manner during the critical period of synaptic refinement within the CN. Postsynaptic P2X2/3Rs increase the efficacy of the immature endbulb of Held–BC synapse, likely promoting synaptic strengthening during development.

Superior Olivary Complex

Several studies have addressed the potential role of purinergic signaling in the nuclei of the superior olivary complex (SOC). In the MNTB, large principal neurons (Fig. 3) are intermingled with fibers of the trapezoid body [174]. Each neuron receives excitatory synaptic input from a single globular BC of the contralateral CN through a giant axosomatic terminal, the calyx of Held [175], as well as through small conventional glutamatergic inputs from yet unidentified sources [176, 177]. In addition, the principal neurons receive inhibitory inputs that arise in the ventral nucleus of the trapezoid body and the MNTB itself [178, 179], but the respective impact of these projections is still a matter of debate [180–182]. The MNTB neurons also express purinergic receptors [37, 38, 160], which seem to be involved in the early postnatal development of excitatory and inhibitory signal transmission as shown both by *in vitro* and *in vivo* experiments [46, 48, 183, 184]. In the MNTB of prehearing gerbils, ATP γ S evokes depolarization and P2XR-mediated currents in ~50% of neurons [46]. The ATP γ S-evoked currents are sensitive to TNP-ATP, a specific blocker of P2X1, P2X3, and P2X2/3 receptors. Furthermore, in 11% of the MNTB neurons recorded just after hearing onset, ATP γ S significantly increases both the spontaneous and acoustically evoked firing rates *in vivo*. In addition, ATP γ S application increases the frequency of spontaneous excitatory and/or inhibitory postsynaptic currents in MNTB neurons of the rat recorded around hearing onset [48]. These results suggest an ATP-mediated facilitation of transmitter release at non-calyceal inputs. Detailed physiological examination revealed the presence of P2X3 receptor subunits (likely as a component of the P2X2/3R heteromer) on cell bodies and/or axons of the excitatory non-calyceal inputs [48]. Additional P2X1 subunits (possibly as part of the P2X1/2R) have been postulated to exist on presynaptic inhibitory terminals, whereas P2YR-mediated effects at the calyx-MNTB neuron synapse can be excluded [48].

There are also reports of a functional role of P1Rs at the calyx terminal [183, 184]. During high-frequency activity, endogenously released adenosine binds to presynaptic A_1 Rs, which in turn leads to the inhibition of presynaptic Ca^{2+} currents, eventually resulting in modest synaptic depression [183, 184]. These effects peak during the first postnatal week and become weaker with postnatal development [183].

The MNTB principal cells, which receive their excitatory input through the calyx of Held synapse, function as fast, sign-inverting relays, involved in the processing of sound source information [185] by providing reliable and well-timed inhibitory inputs to the LSO and MSO (Fig. 3). In addition to the inhibitory projection from the ipsilateral MNTB, the LSO neurons receive direct ipsilateral excitatory inputs from BCs, and the integration of both is the basis for the binaural processing of interaural level differences [186, 187]. Interaural time differences are encoded by bipolar MSO neurons, which receive direct excitatory projections from BCs of both sides [188]. Both the MSO and LSO neurons of adult rats express somatic P2X4 and P2X6 receptor subunits [38]. In the gerbil MSO, however, no purinergic signaling was found at the beginning of the second postnatal week by means of whole cell recording [46]. Yet, under the same conditions, around half of the LSO cells showed apparent P2X currents. A study in P1–P6 mice and rats reported pre- and postsynaptic P2Rs on the BC-LSO synapse [47]. Here, presynaptically bound ATP can increase the miniature EPSC frequency or decrease the miniature EPSC amplitude. Whether these effects are mediated by two distinct subgroups of P2 receptors, perhaps also affecting different types of LSO neurons, remains to be determined. Presynaptic P2XRs, boosting spontaneous synaptic events, have been shown in the dorsal horn of the spinal cord [189], the solitary tract nucleus [190], and the hippocampus [191]. On the contrary, in neurons of the medial habenula and hippocampus, presynaptic P2YR activation has been shown to inhibit spontaneous glutamate release [191, 192]. It is conceivable that different subsets of LSO neurons have different expression patterns of P2X and P2Y receptors, but the physiological importance of this distinction is not yet clear. Postsynaptic P2Rs appear to be P2XRs located either extrasynaptically, or on synapses from non-CN inputs [47], such as inputs from the LNTB or VNTB [193]. The functional importance of these projections is not fully understood, and considering the subtle effects seen in slice experiments, purinergic signaling might play only a minor modulatory role in LSO activity. Yet, it cannot be excluded that we still lack comprehensive knowledge about the role of ATP in the developing superior olivary complex circuit.

Physiological Relevance

Since the seminal work of Hubel and Wiesel in the visual system [194–196] it is well established that specific neuronal activity during critical developmental periods is required to produce high precision and strength of synaptic contacts in adult neuronal networks [197, 198]. Spontaneous neuronal activity can be observed in many systems before the onset of sensory processing, thereafter becoming stimulus-evoked, and thus representing early sensory experience. Temporally correlated spontaneous activity, originating in neighboring receptor cells, seems crucial for the refinement of initially promiscuous synaptic contacts and the generation of precise topographic maps along the afferent sensory pathways [78]. Orderly topographic connections from the HC sensors, via hierarchically organized CNS centers, to specific cortical areas represent the fundamental principle of the tonotopic organization in the auditory system [199]. Similar to the somatosensory and visual systems [200], patterned spontaneous activity in the afferent auditory pathway is important for the development of an orderly central nuclear organization, here tonotopy [82]. In the pre-hearing cochlea, groups of 5–10 neighboring (i.e. tonotopically similar) IHCs correlate the firing of SGN neurons projecting to CNS [41, 201]. This synchronous IHC activity contributes to the development of central synapses, but it is also important for maturation of the IHC itself. Extracellular ATP seems to play a central role in driving the Ca^{2+} waves that coordinate the activity of IHCs [96]. In addition, purinergic signaling drives the refinement of OHC–SGN type II synapses by increasing and synchronizing OHC activity [44]. Impairment of ATP-induced Ca^{2+} waves reduces the number of ribbon synapses and afferent fibers on OHCs [44]. This may render type II SGNs silent, since they require concerted input of many OHCs for their activation. Prehearing bursting activity is important for axonal distribution, pruning, and synapse formation between AN fibers and CN neurons [202]. Similar to activity modulation in the cochlea, endogenous ATP release in the CN increases the fidelity of immature endbulb of Held–BC synapses. This effect, mediated by P2X2/3Rs may contribute to synaptic strengthening through Hebbian-like plasticity [203]. P2XRs and NMDARs are similar in terms of relative Ca^{2+} permeability, and both receptor types have been shown to be involved in Ca^{2+} -driven plasticity processes [204]. However, activation of P2XRs can mediate a substantial Ca^{2+} influx at resting membrane potential [205], as opposed to NMDARs. The intracellular Ca^{2+} can be further increased through the P2Y₁R-induced PLC-IP₃- Ca^{2+} pathway [36]. Generally, both synaptic potentiation and depression depend on cytoplasmic Ca^{2+} signals

[163, 206], which can cause bidirectional changes in synaptic strength [207, 208]. Hence, it is conceivable that synaptic pruning or strengthening of immature endbulb–BC synapses depends on ATP-mediated signaling.

Perspectives

Although recent electrophysiological and immunohistological studies in the developing auditory system have provided new mechanistic insights into the functional roles of ATP signaling, there are several aspects which remain to be elucidated. Significant progress has been made in understanding the effects of ATP on the physiology of developing hair cells. The current hypothesis proposes an indirect effect of ATP through the Ca^{2+} -dependent activation of TMEM16A channels expressed on ISCs and the subsequent increase in $[\text{K}^+]_e$ [96, 101]. Local rapid changes of $[\text{K}^+]_e$ promote the coordinated activity of neighboring IHCs and SGNs, causing synchronous bursting of central auditory neurons within respective isofrequency zones, thus potentially providing cues for the refinement and maturation of central circuits [59, 96, 101, 102]. However, what causes the initial spontaneous release of ATP from ISCs, and what is the potential role of P2Rs expressed on IHCs, remains elusive. Furthermore, the recent finding that Ca^{2+} waves, initiated and boosted by ATP in the greater epithelial ridge, travel to the lesser epithelial ridge, where they affect the activity of OHCs [44], raises the question of the functional importance of spontaneous AP firing in OHCs. Does it drive the refinement of OHC afferent innervation, or is it required for the maturation of SGNs and their central projections? Given that early in development both SGN type I and type II send afferent projections to OHCs, it remains intriguing to what extent the OHC activity contributes to the early bursting of the AN. Surprisingly, bursting activity persists in $\text{VGlut3}^{-/-}$ mice that show no glutamate release from IHCs [59]. Apparently, SGNs deprived of synaptic excitation become hyperexcitable, and fire bursts of APs in response to the K^+ efflux from the ISCs [59]. Whether P2Rs, expressed on both IHC–SGN synapses and SGN somata, might contribute to the hyperexcitability of SGNs is an open question.

Among the central auditory neurons, purinergic enhancement of synaptic efficacy at the immature endbulb of Held–BC synapse in the CN has been well characterized [46, 159]. Still, the potential source and the mechanism of ATP release remain unknown. In addition, a better understanding of the development of auditory function would be gained from an animal model lacking purinergic modulation. Furthermore, the functional contribution of purinergic signaling in the SOC circuits is still

inconclusive. Current studies indicate rather mild modulatory effects on transmitter release [46–48, 183, 184], yet the functional impact on sound signal processing is still unknown. Future studies need to reveal whether purinergic signaling in third-order neurons plays such a prominent role as in the developing cochlea and CN neurons. Finally, higher auditory structures, such as midbrain (inferior colliculus), diencephalon (medial geniculate body), and auditory cortex have not yet been investigated regarding the potential contribution of ATP signaling to the development or function of the mature system.

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