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Mini-Review

Corelease of Two Functionally Opposite Neurotransmitters by Retinal Amacrine Cells: Experimental Evidence and Functional Significance

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The Dale's law postulates that a neuron releases the same neurotransmitter from all its branches. In the case of mulitple neurotransmitters it would require all transmitters to be released from all branches. The retinal cholinergic amacrine cells contain and release γ-aminobutyric (GABA) and, therefore, if GABA and acetylcholine (ACh) are released at the same sites, this could mean that amacrine cells simultaneously excite and inhibit postsynaptic cells. Conversely, if the two neurotransmitters are released at different synapses, or if their release is regulated in a distinct manner, they may play different physiological roles. Recent studies carried out in cultured cholinergic amacrine-like neurons showed that Ca^{2+}-dependent release of ACh and GABA have a different sensitivity to membrane depolarization, to the effect of blockers of voltage gated Ca^{2+} channels (VGCC) and to the effect of presynaptic A_1 adenosine receptors. Therefore, it is proposed that in retinal amacrine cells the Ca^{2+}-dependent release of ACh and GABA occurs at distinct cellular locations. The possible nature of these release sites and the physiological significance of this model are discussed in this review. J. Neurosci. Res. 58: 475–479, 1999.

Key words: acetylcholine; GABA; cotransmission; directional selectivity

INTRODUCTION

Neurons may release more than one transmitter. The earlier studies on cotransmission suggested that a given neuron may release one fast transmitter and a slower transmitter(s); differences in the firing rate of neurons may cause a differential release of costored transmitters (Kupfremann, 1991). More recently, it was found that two fast inhibitory neurotransmitters, γ-aminobutyric acid (GABA) and glycine, may be costored and released from the same vesicles in spinal cord neurons (Jonas et al., 1998). A new concept in terms of synaptic function emerged with the discovery of neurons that store and release fast excitatory and inhibitory neurotransmitters. In cultured spinal neurons, ATP is coreleased with GABA (Jo and Schlichter, 1999), and certain retinal amacrine cells release GABA and acetylcholine (Brecha et al., 1988; Vaney and Young, 1988; O'Malley and Masland, 1989; O'Malley et al., 1992; Neal et al., 1992; Santos et al., 1998a). We will review here the current knowledge concerning cotransmission by cholinergic amacrine cells, and its possible physiological significance.

Retinal Amacrine Cells

Retinal amacrine cells are a structurally and functionally diverse group of interneurons that modulate the flow of visual information from the photoreceptors towards the ganglion cells which form the optic nerve. The cell bodies of amacrine cells may be located in the inner nuclear layer (INL) or in the ganglion cell layer of the retina (Wässle and Boycott, 1991). Although amacrine cells may contain several neurotransmitters (Wässle and Boycott, 1991), immunocytochemical and biochemical studies have shown that, in the retina, acetylcholine (ACh) is used as a neurotransmitter exclusively by a subpopulation of amacrine cells, called starburst amacrine cells (Masland et al., 1984; Voigt, 1986). The processes of cholinergic amacrine cells with cell bodies in the INL (OFF subtype) form a narrow stratum in the outer part of the inner plexiform layer (IPL), whereas those of

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displaced cholinergic cells (ON subtype) stratify in the inner part (Tauchi and Masland, 1985; Voigt, 1986).

The elongated processes of cholinergic amacrine cells form a dense network, radially and symmetrically distributed in the IPL, receiving direct inputs from cone bipolar cells and other amacrine cells (Famiglietti, 1983; Brandon, 1987). The inputs from cone bipolar cells are received throughout the dendrites, but the release of ACh occurs at the varicosities located in the distal portion of the dendrites (Famiglietti, 1983, 1987; Brandon, 1987; Grzywacz et al., 1987). The outputs of cholinergic amacrine cells are directed primarily to complex ganglion cells, including the directionally selective ganglion cells (Masland and Ames, 1976; Ariel and Daw, 1982a,b). Originally, it was proposed that starburst amacrine cells do not propagate action potentials, suggesting that different dendrites could behave independently, but this hypothesis is not supported by the most recent data (e.g., Velte and Miller, 1997).

A subpopulation of cholinergic amacrine cells can also store and release another fast-acting neurotransmitter, GABA (Brecha et al., 1988; Vaney and Young, 1988; O'Malley and Masland, 1989; O'Malley et al., 1992; Neal et al., 1992; Santos et al., 1998a). Therefore, if GABA and ACh are released at the same sites, this could mean that cholinergic neurons simultaneously excite and inhibit postsynaptic cells. The activation of presynaptic GABA autoreceptors could also modulate the release of ACh. Conversely, if the two neurotransmitters are released at different synapses, or if their release is regulated in a distinct manner, they may play different physiological roles. We will review here the available data concerning the release of the two transmitters by amacrine cells, which support the latter hypothesis.

ACh Release From Amacrine Cells and Cholinergic Receptors in the Retina

Since the starburst amacrine cells are the only cholinergic neurons present in the retina (Voigt, 1986), the release of ACh by these cells has been studied mainly in the intact retina (e.g., Masland et al., 1984; Cunningham and Neal, 1985; O'Malley and Masland, 1988; O'Malley et al., 1992). These cells receive input from the glutamatergic cone bipolar cells, and both N-methyl-D-aspartic acid (NMDA) and kainate stimulate the release of ACh from the rabbit retina (Cunningham and Neal, 1985; Linn and Massey, 1991; Linn et al., 1991). However, NMDA receptors do not mediate the release of ACh evoked by diffuse light (Linn and Massey, 1991). The release of ACh from the rat retina is triggered by Ca^{2+} entry through P- or Q-type voltage-gated Ca^{2+} channels (VGCC), or both, and by as yet uncharacterized Cd^{2+}-sensitive channels (Tamura et al., 1995). In contrast, the KCl-evoked release of radiolabelled ACh from cultured chick amacrine neurons is dependent on Ca^{2+} entry through L- and N-type VGCC, and through as yet uncharacterized channels (Santos et al., 1998a,b).

Both nicotinic (nAChR) and muscarinic (mAChR) receptors for acetylcholine have been demonstrated in the retina, first by using pharmacological methods (e.g., Glickman et al., 1982), and more recently, their cellular distribution has been revealed by in situ hybridization and immunohistochemistry techniques. In the latter studies, carried out mostly in the chick retina, several α and β subunits of nAChR were found in amacrine cells and ganglion cells (Keyser et al., 1993; Hamassaki-Britto et al., 1994). The chick retina bipolar cells were also found to express the α8 nAChR subunit (Keyser et al., 1993). Detailed knowledge of retinal cell types containing specific mAChR has been acquired for the chick retina. In this preparation, the mAChR2 is expressed in numerous amacrine and ganglion cells, the mAChR3 is found in many bipolar cells and some of the amacrine cells, and the mAChR4 is present in the majority of the amacrine and ganglion cells (Fischer et al., 1998). In the rat retina, the mAChR2 was detected mainly in regions away from the cholinergic terminals, suggesting that ACh may not only act as a classical fast neurotransmitter in the retina, but may also act like a hormone (Wassélius et al., 1998). Therefore, these receptors appear not to be autoreceptors in rat amacrine cells (Wassélius et al., 1998). It remains to be determined whether this is also the case for the other mAChR.

Abbreviations

ACh	acetylcholine
AMPA	S-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
GABA	γ-aminobutyric acid
INL	inner nuclear layer
IPL	inner plexiform layer
mAChR	muscarinic acetylcholine receptors
nAChR	nicotininc acetylcholine receptors
NMDA	N-methyl-D-aspartic acid
VGCC	voltage-gated Ca^{2+} channels.

GABA Release From Amacrine Cells and GABA Receptors in Neighbouring Cells

The fact that GABA is a the neurotransmitter of subpopulations of amacrine cells, as well as horizontal cells (Wässle and Boycott, 1991), has made it difficult to investigate the mechanisms controlling GABA release in the intact retina. Therefore, with few exceptions (e.g., Osborne and Herrera, 1994), most of the studies concerning the release of GABA from amacrine cells have been carried out in cultures enriched in chick amacrine cells. In

this preparation, the release of [^{3}H]GABA evoked by KCl occurs by two different processes: (1) a Ca^{2+}-dependent mechanism, possibly by exocytosis, mainly due to Ca^{2+} entry through L-type VGCC, but also involving influx of Ca^{2+} through N-type VGCC ; and (2) a Ca^{2+}-independent mechanism, due to reversal of the plasma membrane GABA transporter (Hofmann and Möckel, 1991; Duarte et al., 1992, 1993; Ferreira et al., 1994; Alfonso et al., 1994; Santos et al., 1998a). Accordingly, the processes and cell bodies of rat amacrine cells were found to possess immunoreactivity for the GABA transporters, GAT-1 and GAT-3, suggesting that the Ca^{2+}-independent release of GABA may occur both at synaptic regions and cell bodies (Johnson et al., 1996).

In the intact retina, the GABAergic amacrine cells are stimulated by glutamate released by bipolar cells (Wässle and Boycott, 1991). Using immunohistochemistry and antisera against GABA, it was possible to visualize the release of GABA from amacrine cells of the rabbit retina in response to stimulation with the glutamate receptor agonists NMDA, S-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate (Osborne and Herrera, 1994). The NMDA or AMPA stimulates the release of [^{3}H]GABA from cultures enriched in amacrine neurons by a Ca^{2+}-independent mechanism, whereas the release evoked by kainate is exclusively due to the reversal of the GABA transporter (Ferreira et al., 1994). Interestingly, the AMPA/kainate receptor agonist, domoic acid, stimulates the release of [^{3}H]GABA in a Na^+-free medium, suggesting that the Ca^{2+} entry through the receptor associated channel may also trigger neurotransmitter release in cultured chick amacrine neurons (Alfonso et al., 1994).

The distribution of GABA receptors in the mammalian retina has been investigated in great detail, and has been reviewed elsewhere (Lukasiewicz and Shields, 1998). Most of the retinal neurons express ionotropic $GABA_A$ receptors, which can be either pre- or postsynaptic. Immunocytochemistry studies, using antibodies against $GABA_A$ receptor subunits and against choline acetyltransferase, revealed that $GABA_A$ receptors are expressed by two populations of cholinergic amacrine cells, present in the ON-stratum and OFF-stratum of the IPL (Wässle et al., 1998). There is also ultrastructural evidence for GABAergic amacrine cells input to ganglion cells (e.g., Chun, and Wässle, 1989), and a single ganglion cell may impress $GABA_A$ receptors with more than one subunit composition (Koulen et al., 1996; Wässle et al., 1988). The other class of ionotropic GABA receptors, the $GABA_C$ receptors, are clustered at postsynaptic sites in the mammalian retina, at the rod and cone bipolar axon terminals (Koulen et al, 1998a). The metabotropic $GABA_B$ receptors are present presynaptically at synapses from GABAergic amacrine cells onto both cone and rod bipolar cell axon terminals, supporting the physiological findings of a presynaptic action of $GABA_B$ receptors in these amacrine cells. $GABA_B$ receptors are also found postsynaptically on amacrine and ganglion cells (Koulen et al., 1998b). However, $GABA_B$ receptors do not affect directly the release of ACh from amacrine cells (Slaughter, 1995), suggesting that in those amacrine cells containing ACh and GABA, the release of the two transmitters is controlled differently by $GABA_B$ receptors.

Corelease of ACh and GABA by Amacrine Cells

Based on studies using intact rabbit retinas, it was proposed that the cholinergic amacrine cells, releasing simultaneously ACh and GABA, release ACh primarily by exocytosis and release GABA by reversal of the neurotransmitter transporter (O'Malley and Masland, 1988; O'Malley et al., 1992). According to this model, the difference in the Ca^{2+} sensitivity of ACh and GABA release in the rabbit retina may constitute the mechanism by which cholinergic cells could physiologically distinguish the release of the two coaccumulated neurotransmitters. However, more recently, it was shown that rat and rabbit amacrine cells also release GABA by a Ca^{2+}-dependent mechanism (Neal et al., 1992), and similar results were reported in cultured chick amacrine cells (Duarte et al., 1992; Santos et al., 1998a). Therefore, it is likely that a different mechanism is involved in the selectivity of the synaptic effects of ACh and GABA coreleased by cholinergic amacrine neurons.

Cultures of chick and rat amacrine neurons are particularly valuable in investigating cotransmission by cholinergic amacrine cells, since they are highly enriched in this cell type (Carvalho et al., 1998; Santos et al., 1998a,c). These cells in culture develop a multipolar morphology like the amacrine cells present in the intact retina, and immunocytochemistry experiments revealed a punctate distribution of the synaptic vesicle marker synaptophysin along their processes, which is likely to correspond to presynaptic specializations (Santos et al., 1998b). In the chick cultures, most of the cells (81%) are cholinergic, as determined by immunocytochemistry, using an antibody against choline acetyltransferase, and the majority (87%) of the GABAergic cells are also cholinergic (Santos et al., 1998a). The early studies performed in the intact rabbit retina had indicated that the synaptic vesicles containing ACh did not contain GABA, showing that indeed the two transmitters would be stored in different synaptic vesicles (O'Malley and Masland, 1989). The Ca^{2+}-dependent release of the two neurotransmitters measured in cultures enriched in chick amacrine cells was found to have a different sensitivity to KCl depolarization, and whereas the release of [^{14}C]GABA was controlled mainly by Ca^{2+} influx through L-type VGCC, the release of [^{3}H]ACh was mostly dependent on

the influx of Ca^{2+} through N-type VGCC (Santos et al., 1998a). Also, the release of [^{3}H]ACh from cultured chick amacrine cells is tonically inhibited by presynaptic adenosine A_1 autoreceptors (Santos et al., 1998b), whereas the release of [^{3}H]GABA is insensitive to adenosine (authors unpublished observations). These results suggest that the Ca^{2+}-dependent release of the two transmitters occurs at different cellular locations, where the Ca^{2+} channels present in the active zones are distinct, and where presynaptic receptors controlling neurotransmitter release are also different.

The output synapses from the cholinergic amacrine cells are made by the varicosities of the distal dendrites (Wässle and Boycott, 1991), but it remains to be established whether all such output synapses release ACh and GABA, at distinct active zones, or whether they have an interdigitating spatial arrangement (Koch et al., 1983). The coexistence of ACh with GABA has been shown in other neurons (Brashear et al., 1986; Beaulieu and Somogyi, 1991). Interestingly, we have recently found that KCl depolarization also stimulates the release of ATP from cultures enriched in cholinergic amacrine cells, suggesting that these cells may also release ATP as a neurotransmitter (Santos et al., 1999). A recent report has shown that the excitatory neurotransmitter ATP and inhibitory neurotransmitter GABA are coreleased as fast transmitters from cultured spinal neurons (Jo and Schlichter, 1999). However, the significance of cotransmission in these cells remains to be established.

Functional Significance of Corelease of ACh and GABA

Direction-selective (DS) ganglion cells of the retina fire a series of action potentials to a bar of light moving across the retina in one ''preferred'' direction, while movements in the opposite ''null'' direction evoke little or no response (Cohen and Miller, 1995; for review see Amthor and Grywacz, 1993). The dendrites of DS-ganglion cells are closely associated with cholinergic amacrine cells (Vaney, 1990), and pharmacological experiments have shown that directional selectivity relies on both cholinergic and GABAergic synaptic transmission (Wyatt and Daw, 1976; Ariel and Daw, 1982a,b; He and Masland, 1997; Grzywacz et al., 1998). According to one model, the directional selectivity is explained based on an asymmetric GABAergic input with symmetric nicotinic cholinergic (from starburst amacrine cells) and glutamatergic inputs (from bipolar cells) to the DS cells (He and Masland, 1997). The other model is identical, except that it proposes that the nicotinic input is also asymmetric (Grzywacz et al., 1998a,b). The most recent experimental evidence favours the two-asymmetric-pathways model of directional selectivity, which postulates that directionally selective responses in the retina can be built from an asymmetric nicotinic input, that provides preferred-direction facilitation, and from an asymmetric GABA input, that provides null-direction inhibition (Grzywacz et al., 1998a,b). Although the exact nature of the ACh and GABA-mediated asymmetries is not presently understood at the subcellular level, the amacrine cells containing simultaneously ACh and GABA may be specialized from the morphological and functional point of view to perform those functions.

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