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Cotransmission of Acetylcholine and GABA

Adam J Granger¹, Nicole Mulder¹, Arpiar Saunders¹, and Bernardo L Sabatini¹

¹Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston, MA, USA

Abstract

Neurons that produce acetylcholine (ACh) are positioned to broadly influence the brain, with axonal arborizations that extend throughout the cerebral cortex, striatum, and hippocampus. While the action of these neurons has typically been attributed entirely to ACh, neurons often release more than one primary neurotransmitter. Here, we review evidence for the cotransmission of the inhibitory neurotransmitter GABA from cholinergic neurons throughout the mammalian central nervous system. Functional cotransmission of ACh and GABA has been reported in the retina and cortex, and anatomical studies suggest that GABA cotransmission is a common feature of nearly all forebrain ACh-producing neurons. Further experiments are necessary to confirm the extent of GABA cotransmission from cholinergic neurons, and the contribution of GABA needs to be considered when studying the functional impact of activity in ACh-producing neurons.

1. Introduction

It has been recognized for decades that neurons in the mammalian central nervous system may release both a fast-acting, typically amino acid derived neurotransmitter such as glutamate or GABA, and a second peptidergic neuromodulatory molecule such as neuropeptide Y, substance P, or cholecystokinin. Typically, the fast-acting neurotransmitter is released in response to a single action potential and its effect via activation of ionotropic receptors on the membrane potential of a postsynaptic neuron is easily detected. In contrast, although peptide expression often demarcates distinct classes of intermingled neurons, such as somatostatin (SST) vs. vasoactive intestinal polypeptide (VIP) expressing cortical interneurons (Rudy et al., 2011) or dynorphin vs. enkephalin expressing striatal projection neurons (Steiner and Gerfen, 1998), the patterns of activity that trigger peptide release and the physiological effects of peptide-transmission remain unclear. Thus for most neurons, the relationship between pre and postsynaptic activity is only understood for a single primary neurotransmitter.

The ability to optogenetically activate genetically-defined cell types has led to a growing appreciation that mammalian neurons can release multiple fast-acting neurotransmitters (Hnasko and Edwards, 2012; Vaaga et al., 2014), subverting the classic notion that neuron

Address all correspondence to: Bernardo Sabatini, bernardo_sabatini@hms.harvard.edu.

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classes can be defined by their ability to package and release a single primary neurotransmitter. Examples include spinal interneurons that release GABA and glycine (Jonas et al., 1998), midbrain dopaminergic neurons that also release glutamate and/or GABA (Stuber et al., 2010; Tecuapetla et al., 2010; Tritsch et al., 2012, 2014), and habenula-projecting neurons that release both GABA and glutamate (Root et al., 2014; Shabel et al., 2014).

In the central nervous system, acetylcholine (ACh) and the activity of cholinergic neurons has been shown to facilitate learning and memory formation, increase alertness and attention, and signal behaviorally relevant sensory cues (Sarter et al., 2009; Hasselmo and Sarter, 2011; Picciotto et al., 2012). These behavioral functions are mediated by multiple populations of neurons that produce and release ACh. However, the specialized functions of each ACh-releasing population are largely unknown. In the cerebral cortex, ACh is released from long-range axons projecting from basal forebrain neurons (Jones, 2004), with a potential contribution from local cholinergic interneurons (von Engelhardt et al., 2007; Consonni et al., 2009; Cauli et al., 2014). Like the cortex, the striatum has both local cholinergic interneurons and long-distance innervation from the brainstem cholinergic centers (Calabresi et al., 2000). The hippocampus and neighboring entorhinal cortex, however, are innervated by extrinsic cholinergic inputs from the medial septum and horizontal and diagonal limbs of the band of Broca (MSDB; Teles-Grilo Ruivo and Mellor, 2013) and by intrinsic cholinergic interneurons (Frotscher et al., 1986, 2000; Romo-Parra et al., 2003; Yi et al., 2015). In these systems, ACh can activate ionotropic, excitatory nicotinic acetylcholine receptors (nAChRs) or metabotropic muscarinic acetylcholine receptors (mAChRs). These receptors then regulate postsynaptic cellular excitability and the synaptic release of other neurotransmitters from the presynaptic cell, which can alter synaptic plasticity (Picciotto et al., 2012; Arroyo et al., 2014).

In the forebrain, the first identified co-transmitter to be released with ACh was glutamate. VGLUT3, a vesicular glutamate transporter, is expressed by subpopulations of cholinergic neurons, and, in the striatum, is necessary for glutamate release from striatal cholinergic interneurons (Higley et al., 2011). Interestingly, serotonergic neurons that innervate the striatum also express VGLUT3 (Gras et al., 2002) and release glutamate in the hippocampus (Varga et al., 2009). The functional consequences of VGLUT3-dependent glutamate release from these neurons is not fully understood and the protein may facilitate in vesicular loading of glutamate as well as of acetylcholine and monoamines (El Mestikawy et al., 2011). Little else is known about glutamate corelease from cholinergic neurons, and for the remainder of this mini review, we focus on the evidence for GABA and ACh cotransmission throughout the nervous system and speculate on potential functional consequences.

2. Functional demonstrations of ACh/GABA cotransmission

The cotransmission of GABA and ACh has been best characterized in starburst amacrine cells (SACs) of the retina. SACs are a subtype of amacrine cell, a diverse set of retinal interneurons that are crucial for processing visual information. SACs are the sole source of acetylcholine in the retina (Masland et al., 1984; Voigt, 1986), and are characterized by their radial dendrites and synaptic innervation of direction-sensitive retinal ganglion cells

(DSGCs), which are most active when a light stimulus is presented moving in the cell's preferred direction (Duarte et al., 1999). Immunohistochemical analyses showed that SACs also contain and release GABA (Brecha et al., 1988; Kosaka et al., 1988a; Vaney and Young, 1988; O'Malley and Masland, 1989; Santos et al., 1998). Indeed, the activation of DSGCs by moving light stimulation requires GABA receptors, suggesting an important role for GABA release from SACs in establishing direction selectivity (Wyatt and Day, 1976; Caldwell et al., 1978). The functional relevance of ACh release is less clear. Recently, Lee and colleagues (Lee et al., 2010) definitively demonstrated monosynaptic transmission by both ACh and GABA between SACs and DSGCs using paired recordings from whole-mount rabbit retina. Release of each neurotransmitter was differentially sensitive to conditions of either low external Ca^{2+} or blockers of voltage-gated calcium channels – ACh-mediated transmission was blocked by low external Ca^{2+} concentration and antagonists of N-type Ca^{2+} channels, whereas GABA transmission was relatively unaffected. This indicates release from separate populations of synaptic vesicles. In addition, GABA release was spatially restricted onto DSGCs dendrites that were on the side of that DSGC's null direction, and could only be evoked by a light stimulus moving in the DSGC's null direction. Release of ACh, in contrast, showed no such spatial preference and could be evoked by light stimuli moving in any direction. This example of cotransmission demonstrates that differential packaging and release of multiple fast-neurotransmitters can be used to serve unique circuit functions.

In the CNS, we have recently described an instance of functional ACh and GABA cotransmission onto interneurons in cortical layer 1 (Saunders et al., 2015a). Selective activation of cholinergic axons in cortex using channelrhodopsin (ChR2) evoked both ACh-mediated excitatory postsynaptic currents (EPSCs) and GABA-mediated inhibitory postsynaptic currents (IPSCs) in layer 1 interneurons. A portion of the GABA_A receptor mediated IPSCs was blocked by nicotinic antagonists, and therefore appear to be a result of feed-forward inhibition due to nicotinic activation of intermediary interneurons. However, a fraction remained and exhibited short onset latency that was consistent with direct release of GABA from cholinergic axons. These IPSCs persisted also after co-application of tetrodotoxin and 4-AP (Petreanu et al., 2009). The rapid onset, resistance to block by nicotinic antagonists, and independence from action potential firing all indicate direct release of GABA from cholinergic fibers. Recordings from mice where the vesicular GABA transporter (VGAT, encoded by *slc32a1*) was selectively deleted from cholinergic neurons further confirmed this finding. In these mice, ACh-mediated EPSCs were unaffected, but activation of cholinergic fibers could no longer elicit the low-latency IPSCs, ruling out a role for an intermediary neuron population acting as the source of GABA.

Unlike in SACs, it is unknown whether ACh and GABA are released in cortex from the same or different sets of synaptic vesicles. The proportion of layer 1 interneurons that receive GABAergic or cholinergic inputs differ significantly, with many cells receiving one of the inputs but not the other (Saunders et al., 2015a). This is suggestive of separate vesicle populations for each neurotransmitter, but could also be explained by differences in postsynaptic expression of nAChRs and GABA receptors. Additional evidence for separate vesicle populations comes from a particular projection between the globus pallidus externus

and frontal cortex, which includes cholinergic neurons that release of both ACh and GABA (Saunders et al., 2015b). Serial fluorescent immunostaining of ultrathin sections by array tomography revealed physically separate sites of labeling of VGAT and the vesicular ACh transporter (VACHT), encoded by *slc18a3*, even when both transporters were detected in the same terminal. This suggests that, at least within this particular cholinergic projection, ACh and GABA release likely occurs through release of separate vesicular pools.

3. Colabeling evidence for ACh and GABA cotransmission

Though functional demonstrations of ACh/GABA cotransmission remain largely limited to the retina and our recent analyses of cortex (Lee et al., 2010; Saunders et al., 2015a, 2015b), evidence suggestive of ACh/GABA cotransmission has long existed in the literature. The earliest evidence comes from the chick ciliary ganglion. Neurons in this ganglion receive input through a large and reliable calyx-type synapse that depolarizes the ganglion neurons via electrical synapses and by activating nicotinic acetylcholine receptors following ACh release in the calyx (McEachern et al., 1985). However, numerous studies have shown that these neurons are also sensitive to exogenous GABA, through the opening of GABA_A receptors. With no local interneurons and the only input coming from the cholinergic neurons of the accessory oculomotor nucleus (AON), this suggests that the presynaptic neurons of the AON may cotransmit ACh and GABA (McEachern et al., 1985; Engisch and Fischbach, 1990, 1992). However, the dual cholinergic/GABAergic identity of these neurons has not been confirmed and, to our knowledge, coexpression of ACh and GABAergic synthetic and packaging enzymes has not been examined in the chick AON.

Immunolabeling of neurotransmitters or proteins necessary for neurotransmitter synthesis and packaging had also hinted that cholinergic neurons of the rodent basal forebrain may be GABAergic. Kosaka and colleagues examined consecutive 40 micron fixed tissue sections that transected individual neurons, staining one section with choline acetyltransferase (ChAT), the enzyme that synthesizes ACh, and the other with glutamate decarboxylase (GAD), the enzyme that synthesizes GABA, or GABA itself. They reported individual neurons immunopositive for both sets of markers in the basal forebrain, retina (most likely corresponding to the SACs), as well as the spinal cord (Kosaka et al., 1988b). Another study confirmed this finding of overlapping expression of ChAT and GAD in consecutive tissue sections of individual basal forebrain neurons retrogradely labeled with cortically-injected wheat germ agglutinin bound to horse-radish peroxidase (Fisher and Levine, 1989). However, not every study has observed colabeling of GAD and ChAT proteins, instead finding intermingled, non-overlapping populations of GAD- and ChAT-positive neurons (Gritti et al., 1993). One probable explanation for this discrepancy is the differences in subcellular localization of the two major GAD isoforms, GAD65 and GAD67. The GAD67 isoform, which is commonly used as a marker for GABAergic identity, is found throughout the entire cell, including the soma. GAD65, in contrast, is localized in synaptic terminals and largely not detected at the soma (Soghomonian and Martin, 1998). If cholinergic neurons only express GAD65, then somatic immunostaining may miss GAD expression. Indeed, genetic labeling of GABAergic neurons shows overlapping expression of ChAT with neurons that express VGAT (*slc32a1*) and *gad2*, the gene that encodes GAD65, but not *gad1*, the gene for GAD67 (Saunders et al., 2015a). Notably, overlap of ChAT with

GABAergic markers was limited to the forebrain and did not extend to the midbrain pedunculopontine nucleus, which also contains population of ChAT-expressing neurons. Because GAD protein may not always localize in the cell body, a more reliable way to assess if a neuron expresses GAD is to examine mRNA directly. One study using single-cell RT-PCR did show that individual neurons of the basal forebrain (BF) and globus pallidus (GP) do express transcripts of proteins indicative of both GABA release, such as *gad* and *slc32a1*, and ACh release, like ChAT (*chat*) and VAcH (*slc18a3*) (Tkatch et al., 1998).

To directly demonstrate expression of GABA synthetic enzymes in cholinergic neurons, we used fluorescent *in situ* hybridization to visualize expressed mRNA transcripts and thus avoid the confounds of missing protein expression due to non-somatic localization. We designed two probes with the same fluorophore against mRNA for both isoforms of GAD (*gad1* and *gad2*), as well as a probe for *chat*, and performed *in situ* hybridization with tissue sections from adult, wild-type mice. We observed that cell bodies of the globus pallidus and nucleus basalis of the basal forebrain fluorescently labeled with probes against ChAT also showed GAD expression, though GAD expression appeared weaker in ChAT⁺ neurons than in neighboring ChAT⁻ neurons (Figure 1). This demonstrates that cholinergic neurons do express the necessary machinery to synthesize GABA for release in adulthood.

Further evidence for ACh/GABA cotransmission had also been observed in the cortex. Extensive axonal arborization from cholinergic BF neurons exists throughout the cortical layers, allowing direct observation of cholinergic synaptic terminals. One study in the visual cortex of cat examined immunolabeling of ChAT and GABA in electron microscopy cryosections, for the purpose of identifying the extent of cholinergic innervations of cortical GABAergic interneurons. This study reported not only an enrichment for cholinergic terminals on GABA-immunoreactive postsynaptic cells, suggesting a preference of cholinergic axons for inhibitory interneurons, but also substantial co-labeling of presynaptic cholinergic terminals for both GABA and ChAT (Beaulieu and Somogyi, 1991). In addition, retrograde labeling of cortically projecting BF neurons with cholera toxin showed co-staining with markers for GABA release (Gritti et al., 1997). While many of these most likely represent a separate population of cortically-projecting GABAergic neurons, they are consistent with cortically projecting ChAT⁺ neurons also expressing GABA.

In addition to receiving long-range cholinergic projections from basal forebrain, the cortex contains a small population of local, apparently cholinergic interneurons. These ChAT-expressing cells express the vasointestinal peptide (VIP), a common marker for a subclass of cortical interneuron, and share similar stereotyped morphology and laminar distribution (Consonni et al., 2009; Cauli et al., 2014). Only one study to date has attempted to directly address the function of these neurons, using paired recordings between cortical ChAT-expressing cells and neighboring pyramidal neurons (von Engelhardt et al., 2007). The authors found that these cells can influence signaling in adjacent pyramidal neurons indirectly by increasing spontaneous EPSCs via presynaptic nicotinic receptors. This study does not directly report a GABAergic output of these intrinsic cortical ChAT cells, but did report that a subset of them express GAD67. It seems likely therefore that these cells also release GABA, either onto a cell-type not well sampled by this study or with sparse

connectivity not easily detected using paired recordings between presynaptic cholinergic neurons and postsynaptic pyramidal neurons.

Beyond analysis of presynaptic markers, colocalization of postsynaptic receptors or scaffolding proteins could suggest individual synapses that are sensitive to both ACh and GABA. For example, in chick ciliary ganglion, ultrastructural analysis shows discrete clusters of both nAChRs and glycine receptors (GlyRs) directly opposing individual presynaptic specializations and overlapping with gephyrin, an important postsynaptic scaffolding molecule for GABA receptors and GlyRs (Tsen, 2000). In cultured hippocampal neurons, nAChRs labeled by fluorophore-conjugated alpha-bungarotoxin cluster around GABAergic synapses, both presynaptically and postsynaptically, and extensive codistribution of nAChRs with GABA receptors can be observed at synapses and developing filopodia (Kawai et al 2002, Zago et al 2006). In the cortex, costaining of labeled basal forebrain axonal projections not only shows presynaptic terminals that contain both VGAT and VAcHT protein, but also adjacent gephyrin-positive postsynaptic specializations abutting ~30% of VAcHT-labeled terminals, suggesting the possibility of dual ACh/GABA synapses (Henny, Jones, 2008). Similarly, GFP-labeled terminals of cortically-projecting cholinergic GP neurons also colocalize with gephyrin, but not with PDS-95 (Saunders et al., 2015b). Despite these promising examples, however, very little ultrastructural evidence exists comparing the relative distribution of ACh and GABA receptors at central synapses which could provide more definitive support for cotransmission.

4. Evidence from Optogenetics

In the past, paired recordings between presynaptic and postsynaptic neurons were the gold standard to unambiguously demonstrate neurotransmitter corelease, ensuring that the different neurotransmitters were originating from the same presynaptic source. However, finding connected pairs of neurons can be very difficult, and this method is not feasible for long-range projections. In contrast, electrical stimulation of axonal fibers, which allows for activation of far more presynaptic cells, increases the chances of observing a postsynaptic response, but cannot distinguish between axons of different cell populations, making it difficult to determine whether distinct neurotransmitters came from the same or different cells. Cre-dependent optogenetic stimulation using ChR2 or its variants can activate many neurons of a genetically-defined subpopulation at once (Zhao et al., 2011), identifying classes of neurons that release multiple neurotransmitters but not proving that any individual neuron does so.

Several studies have recently used optogenetics to selectively activate various cholinergic neurons (Jiang et al., 2014). However, most do not explicitly report direct release of GABA from cholinergic fibers, with the exception of our two studies described above (Saunders et al., 2015a, 2015b). Some do describe results that are consistent with such a phenomenon but that could also be explained by feed-forward inhibition, such as an increase in inhibitory events and decreases in spiking following photo-activation of striatal cholinergic interneurons (Witten et al., 2010) and suppression of mitral/tufted cell firing in the olfactory bulb following photo-activation of cholinergic fibers from the diagonal band of Broca (Ma and Luo, 2012). Others examined the effects of photo-activation in ways that would not

detect GABA release, such as measuring local field potentials or single unit spiking in cortex following activation of BF axons (Pinto et al., 2013), or recording postsynaptic currents in conditions that would explicitly preclude observing GABAergic responses by including GABA receptor antagonists (Higley et al., 2011; Yang et al., 2014). However, several studies report postsynaptic effects that can be entirely blocked by cholinergic antagonists, arguing against a major role for GABAergic signaling (Nagode et al., 2011; Arroyo et al., 2012; Bennett et al., 2012; Kalmbach et al., 2012; Eggermann et al., 2014; Kalmbach and Waters, 2014; Unal et al., 2015). In these cases, perhaps the specifics of recording configuration, such as recording at or near the reversal potential for GABA receptors, may have prevented the observation of clear GABA-mediated responses. Additionally, the use of transgenic mice that target ChR2 to cholinergic neurons using a bacterial artificial chromosome containing the *chat* regulatory regions may also interfere with normal synaptic transmission, as these mice lines exhibit several fold increases in expression of VAcHT (Kolisnyk et al., 2013). No doubt, future studies that combine optogenetic activation of cholinergic neurons with careful monitoring for GABA-mediated effects will be invaluable for determining when and where the cholinergic system is releasing GABA.

5. Sources and targets of GABA/ACh cotransmission

To understand the effects on the circuit function of the brain, we must first understand the sources and targets of GABA release from cholinergic neurons. Though the co-labeling of GABAergic markers like VGAT and GAD65 appears widespread throughout cholinergic neurons of the forebrain, including those of the basal forebrain, MSDB, and cortex (Saunders et al., 2015a), it is unclear whether they all, or only some subsets, actually release GABA. It remains possible that expression of VGAT and GAD does not equate to synaptic release of GABA, but instead are expressed for an alternate developmental or evolutionary reasons. Indeed, studies that have attempted to specifically ablate GABAergic projection neurons of MSDB suggest that this may be the case (Pang et al., 2011; Köppen et al., 2013; Roland et al., 2014). The authors used GAT1-SAP, an immunotoxin combining the ribosome-inactivating protein saporin with an antibody against the membrane GABA transporter GAT1, to selectively target and kill GABAergic projection neurons. They report no decrease in the number of cholinergic neurons, suggesting that GABAergic and cholinergic neurons of the MSDB are separate populations. However, this immunotoxin requires expression of GAT1 to be effective, and GAT1 is not an absolute requirement for GABAergic identity. Still, GABA release from cholinergic neurons needs to be confirmed by electrophysiology for each major cholinergic center of the forebrain to determine the extent of GABA/ACh cotransmission. Given our findings of co-expression of mRNA encoding for GAD and ChAT in adult globus pallidus neurons and of GABAergic and cholinergic currents in cortex evoked by ChR2-mediated activation of these neurons (Figure 1; Saunders et al., 2015b), at least this class of cholinergic neuron mediates a bona fide GABAergic projection as well.

Another open question is whether GABA and ACh are released from the same synaptic vesicles, or even the same presynaptic terminals. Multi-transmitter neurons may either function through co-release, in which multiple neurotransmitters are packaged into the same

presynaptic terminal and are therefore released simultaneously, or co-transmission, in which the different transmitters are packaged separately and may be differentially released (Vaaga et al., 2014). For the purposes of this review, we have referred to ACh and GABA cotransmission, since preliminary results suggests separate pools of synaptic vesicles (Saunders et al., 2015b), and retinal SACs provide one definitive example of cotransmission (Lee et al., 2010). This latter example also demonstrates how release from separate populations of synaptic vesicles allows for spatial or functional compartmentalization of GABA and ACh release. If GABA and ACh are indeed released from the same neuron but under different conditions or from separate locations, this could explain why cotransmission has been previously overlooked in the literature, as functional identification of cotransmission may be difficult to identify.

6. Functional consequences of ACh/GABA cotransmission

At first glance, release of an excitatory (ACh) and inhibitory (GABA) neurotransmitter by the same axons would appear to be functionally antagonistic. However, both transmitters could act in parallel, depending on the mode of cotransmission. If both ACh and GABA are released simultaneously onto the same postsynaptic cells, then the GABA may act to restrict or shunt the level of excitation provided by ACh, similar to the corelease of GABA and glutamate in the habenula (Root et al., 2014; Shabel et al., 2014). Along these lines, release of one neurotransmitter may be used to modulate the subsequent release or response to future bouts of stimulation. For example, nAChRs can influence GABA receptors either postsynaptically by decreasing subsequent inhibition through Ca^{2+} -mediate phosphorylation of GABA receptors, or presynaptically by enhancing evoked GABA release (Shrivastava et al., 2011) and presynaptic metabotropic GABA receptors have been shown to inhibit vesicle release in glutamatergic (though as of yet, not cholinergic) synapses (Chalifoux and Carter, 2011). Cotransmission of ACh and GABA might also target different postsynaptic cells, such that GABA inhibits one cell population while ACh excites another. This could be accomplished through postsynaptic cells only expressing one set of receptors, sensitive to either GABA or ACh, or by differentially releasing GABA or ACh in a target-specific manner.

In cerebral cortex, excitatory ACh and inhibitory GABA actions can still have the same consequence on overall activity – for example, ACh excites disinhibitory layer 1 interneurons, while GABA directly inhibits interneurons in deeper layers (Letzkus et al., 2011; Pi et al., 2013; Fu et al., 2014; Saunders et al., 2015a). In this case, both transmitters would act to disinhibit and increase cortical activity. In conjunction with possible actions via muscarinic receptors to biochemically enhance plasticity induction, this mechanism may explain many of the proposed effects of cholinergic centers on cerebral cortex.

7. Conclusion

Accumulating evidence indicates that mammalian central cholinergic centers likely mediate diverse effects via multiple released neurotransmitters. It is worth considering that the basal forebrain projections to cortex appear to be all GABAergic, and thus one might recategorize this center as a GABAergic projection in which a subset of neurons corelease ACh. Future

genetic and optogenetic studies are needed to distinguish the contribution of each neurotransmitter to the behavioral consequence of activity in these centers. Careful consideration of the experimental conditions for each published study is necessary to evaluate if reported effects are due to ACh or GABA release.

Materials and Methods

Fluorescent in situ hybridization

Fluorescent in situ hybridization was performed according to the protocol provided by ACD RNAscope Multiplex Assay manual. Briefly, mice were deeply anesthetized with isoflurane, decapitated, the forebrain dissected out and frozen in tissue freezing medium (Tissue-Tek) on dry ice. The brain was then sliced with a cryostat (Leica CM 1950) into 30 μ m sections, adhered to SuperFrost Plus slides (VWR), and immediately refrozen. Samples were then fixed in pre-chilled 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at 4°C, dehydrated with 50% (x1), 70% (x1), and 100% (x2) ethanol washes for 5 minutes. Slides were air-dried and a barrier drawn around the tissue section with an Immedge hydrophobic barrier pen (Vector Laboratories) and incubated in pretreatment 3 buffer (ACD) at room temperature for 30 minutes, then rinsed twice in PBS for 5 minutes. Fluorophore-conjugated probes, one complementary to *chat* mRNA (Atto 550, NM_009891.2, region 1090-1952, catalog #408731-C2) and two probes against *gad1* (NM_008077.4, region 62-3113, catalog #400951-C3) and *gad2* (NM_008078.2, region 552-1506, catalog #439371-C3) mRNA (both Atto 647) were incubated with the slide-mounted tissues sections at 40°C in a HybEz oven (ACD) for 2 hours and washed twice in distilled H₂O. Following fluorescence amplification, sections were stained with DAPI and covered with ProLong antifade reagent (Molecular Probes). Confocal images (1-2 μ m optical sections) were acquired with an Olympus FV1200 laser scanning confocal microscope (Harvard Neurobiology Imaging Facility) through a 10 $\times$ air objective and a 40 $\times$ oil-immersion objective.

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Highlights

- The cholinergic system is broadly important for normal brain function, including learning, attention, and cue-detection
- Functional evidence for cotransmission of GABA with Acetylcholine is reviewed from retinalstarburst amacrine cells and cortical cholinergic fibers
- Colabeling of GABAergic markers indicates that much of the forebrain cholinergic neurons mayalso release GABA
- Optogenetic stimulation will allow for definitive determination of cholinergic cells that do or donot release GABA

Figure 1.

ChAT-expressing subcortical neurons also express *Gad1/Gad2*.

A) Sagittal (+2.15 mm from midline) diagram of the mouse brain showing region of interest. Red and blue boxes indicate the areas of interest in (B) and (C), respectively. Str – Striatum, GP – Globus Pallidus, NB – Nucleus Basalis, D – dorsal, V- ventral, R – rostral, C – caudal. B) Low (left) and high (right) magnification example confocal image of the border between GP and NB, demonstrating fluorescent *in situ* hybridization for *chat* (magenta, top) and a combined probe for *Gad1* and *Gad2* (green, middle). Arrow heads indicate the position of ChAT-expressing neurons of the GP (top), with corresponding *Gad1/Gad2* expression (middle). Merged images includes DAPI stain showing cell nuclei (blue, bottom). Insets show magnified view of individual neuron indicated by the asterisk (*).

C) Same as in (B), but showing the NB proper. Scale is consistent with bars indicated in (B).