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Role of endogenous nicotinic signaling in guiding neuronal development

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ABSTRACT

Spontaneous nicotinic cholinergic activity is widespread in the developing nervous system. One of the major components mediating this activity is the nicotinic acetylcholine receptor with α7 subunits (α7-nAChR) and high relative calcium permeability. We recently reported that α7-nAChRs co-localize in part with $GABA_A$ receptors during development, and the sites become co-innervated by cholinergic and GABAergic terminals. Patch-clamp recording either from embryonic chick ciliary ganglion neurons or from early postnatal mouse hippocampal interneurons reveals that α7-nAChR activation can impose a rapid and reversible decrease in $GABA_A$ receptor responses. The effect extends to GABAergic synaptic currents, and depends on intracellular calcium, calcium/calmodulin-dependent protein kinase II, and MAP kinase in the postsynaptic cell. Over the longer term, nicotinic activity has a more profound effect: it determines the time during development when GABAergic signaling converts from excitation to inhibition. It does this by changing the pattern of chloride transporters to establish the mature chloride gradient required for inhibitory GABAergic responses. The excitatory phase of GABAergic signaling is critical for proper development and integration of neurons into circuits. By driving the conversion of GABAergic signaling, nicotinic activity not only terminates one set of developmental instructions, but also initiates another by collaborating with GABAergic inhibition to impose new instructions. The results reveal a multi-layered pattern of activity-dependent controls in development and indicate the significance of nicotinic signaling in shaping these events.

1. Introduction

Nicotinic cholinergic signaling is widespread in the vertebrate nervous system where it employs the transmitter acetylcholine (ACh) from cholinergic neurons to activate ligand-gated cation-selective ion channels [1,2]. Classic studies have demonstrated the excitatory role of nicotinic signaling at the vertebrate neuromuscular junction and in autonomic ganglia. In the central nervous system, however, nicotinic signaling appears to exert more complex effects. Numerous

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Abbreviations: αBgt, α-Bungarotoxin; α7-nAChRs, α7-containing nicotinic acetylcholine receptors; α4β2-nAChRs, α4- and β2-containing nicotinic acetylcholine receptors; ACh, acetylcholine; CG, ciliary ganglion; CaMKII, calcium/calmodulin-dependent protein kinase II; DHβE, dihydro-β-erythroidine; GAD, glutamic acid decarboxylase; GFP, green fluorescent protein; IPSCs, inhibitory postsynaptic currents; MLA, methyllycaconitine; SO, stratum oriens; SR, stratum radiatum; VAChT, vesicular ACh transporter; VGCCs, voltage-gated calcium channels

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studies have demonstrated the ability of nicotinic input to act presynaptically, modulating transmitter release at a variety of synapses [3–11]. Nicotinic input can also act postsynaptically both to generate depolarizing postsynaptic currents and to activate downstream signaling cascades [12–17]. The latter can have diverse effects depending on the location and type of pathway targeted.

A common theme in nicotinic signaling is the engagement of calcium-dependent signal cascades. The two major nicotinic acetylcholine receptors (nAChRs) expressed in the brain are the heteropentameric receptors containing α4 and β2 gene products (α4β2-nAChRs) and the homopentameric receptors containing the α7 gene product (α7-nAChRs). The latter have a high relative permeability to calcium and can directly activate calcium-dependent pathways [18,19]. Though α4β2-nAChRs are less permeable to calcium, they too activate calcium-dependent pathways in part because they are slower to desensitize and recruit contributions from voltage-gated calcium channels (VGCCs).

Spontaneous nicotinic excitation arises early in development and often occurs in bursts or waves traveling through the tissue. This has been well-documented for the retina and spinal cord where it helps direct target selection and synapse formation [20–23]. Spontaneous nicotinic excitation also contributes to the giant depolarizing potentials reported for neurons in the developing hippocampus [9,24,25]. This pervasiveness of nicotinic excitation at late embryonic and early postnatal stages raises questions about the role of nicotinic input in neurodevelopment. The recent studies reviewed here address the interactions of nicotinic and GABAergic signaling and some of the consequences of that interaction for development in the nervous system.

2. Convergence of nicotinic and GABAergic input during development

The two most abundant nicotinic ACh receptors in the central nervous system – α4β2-nAChRs and α7-nAChRs – reach high levels in the early postnatal hippocampus [26,27]. Using fluorescently tagged α-bungarotoxin (αBgt) to visualize α7-nAChRs on the cell surface demonstrates that highest levels of the receptors are to be found on hippocampal interneurons when examined in slice culture [28]. This is consistent with electrophysiological analysis of α7-nAChR responses in fresh hippocampal slices as well as in dissociated cell culture [29–32]. The surprise finding was that the receptors appear to co-cluster in part with $GABA_A$ receptors on the neurons, and moreover, that the co-clusters are often found at the tips of filopodia extending from dendrites on the interneurons. The co-clustering of α7-nAChRs with $GABA_A$ receptors was specific, did not include GluR1-containing glutamatergic receptors, and did not require innervation. When septal explants were co-cultured with the hippocampal slices to provide cholinergic innervation, the α7-nAChR/$GABA_A$ receptor co-clusters became co-innervated by cholinergic and GABAergic terminals. This could be seen by co-staining for α7-nAChRs and vesicular ACh transporter (VAChT) as a marker for cholinergic terminals (Fig. 1A–C) or α7-nAChRs, $GABA_A$ receptors, and glutamic acid decarboxylase (GAD) as a

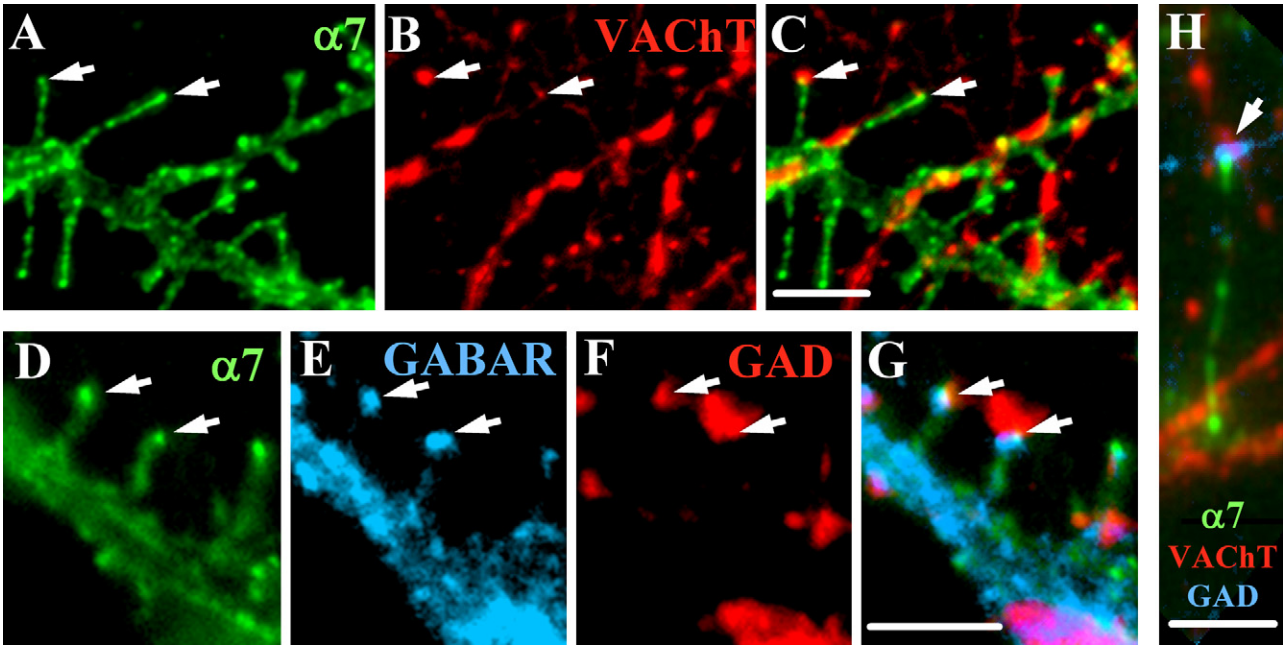

Fig. 1 – Innervation of α7-nAChR filopodia by cholinergic and GABAergic terminals from septum. Three-week-old septal-hippocampal co-cultures were stained for surface α7-nAChRs with AlexaαBgt (A, D and H) and co-stained either for VAChT with antibodies (B) and shown in overlay (C), or co-stained for α1-$GABA_A$R subunit (E) and GAD65 (F) with appropriate antibodies and shown in overlay (G), or co-stained for both VAChT and GAD and shown in overlay (H). Arrows indicate examples of receptor clusters on filopodia being innervated by cholinergic (VAChT-positive) and/or GABAergic (GAD-positive) boutons. Scale bars: 10 μm (from Ref. [28]).

marker for GABAergic terminals (Fig. 1D–G), or α7-nAChRs, VAChT, and GAD (Fig. 1H). Maintenance of the co-innervation required nicotinic activity exerted through α7-nAChRs [28]. Analysis of fresh hippocampal slices prepared from early postnatal rat pups confirmed that the receptor co-clusters occurred in vivo as well. The results indicated that cholinergic input was well positioned during development to exert local postsynaptic effects at GABAergic synapses.

3. Acute regulation of GABAergic signaling by nicotinic input

To test for acute regulation of GABA responses by nicotinic input, we first turned to chick ciliary ganglion (CG) neurons which express large numbers of α7-nAChRs and $GABA_A$ receptors on the cell soma. Application of GABA to freshly dissociated embryonic CG neurons reproducibly generates an inward current as previously described [33]. Incubating the neurons with 20 μM nicotine for 2–3 s prior to the GABA application reduced the GABA-induced whole-cell current about 40% [34]. The reduction reversed within 8 s and was blocked by methyllycaconitine (MLA), an inhibitor of α7-nAChRs. The inhibition could also be blocked by infusing the neuron either with BAPTA to chelate calcium or with KN-93 to block calcium/calmodulin-dependent protein kinase II (CaMKII) and U0126 to block MAP kinase. The results indicated that nicotinic activation of α7-nAChRs, acting through a calcium- and CaMKII/MAPK-dependent mechanism, can reversibly inhibit $GABA_A$ receptors on the same cells [34].

We then prepared hippocampal slices from postnatal days 9–14 mice to test for similar nicotinic effects. Using an extracellular stimulating electrode positioned in the stratum radiatum (SR) of the CA1 region, we could elicit $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) recorded in SR interneurons with a patch-clamp electrode. The antagonists NBQX (20 μM) and APV (50 μM) were used to block AMPA and NMDA receptors, respectively. Application of nicotine from a pipette positioned over the dendritic field (but far from the soma to prevent movement artifacts) reduced the amplitude of IPSCs elicited subsequently by the same stimulation. Again, the effect was blocked by MLA. This suggested that $GABA_A$ receptors on SR interneurons might be regulated by α7-nAChRs as seen for CG neurons.

This hypothesis was tested directly by determining the effects of endogenous cholinergic input. Cholinergic terminals project from the septum to the hippocampus in part via the CA1 region and can be activated by an extracellular stimulating electrode positioned in the stratum oriens (SO). By sequentially pairing stimuli delivered to the SR and SO, one

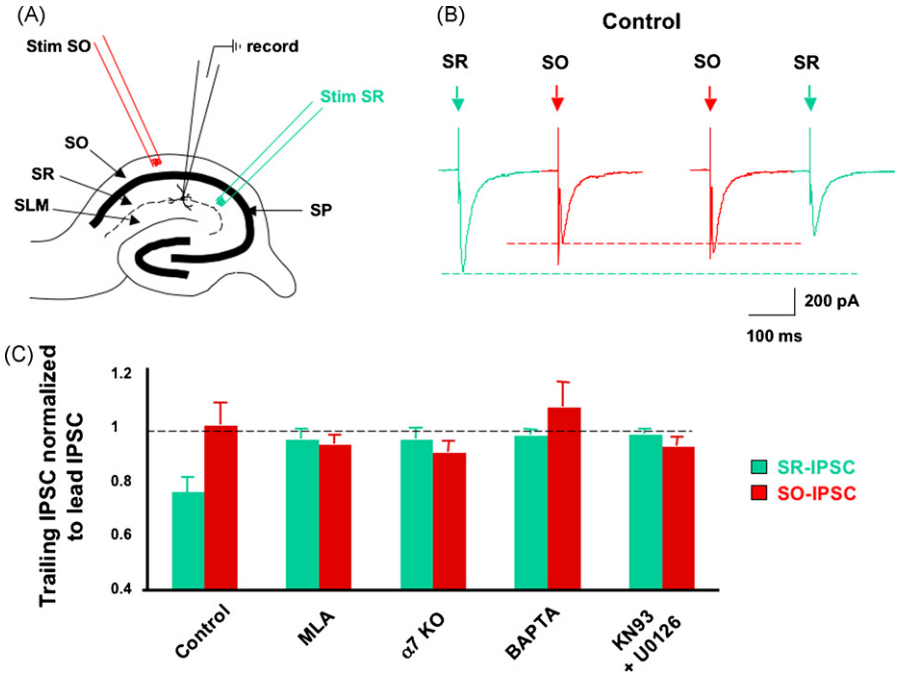

Fig. 2 – SO stimulation to activate cholinergic fibers depresses IPSCs elicited subsequently by SR stimulation. (A) Diagram showing positions of stimulating (Stim) and recording electrodes (Record) in the SO and SR. (B) Two pairs of IPSCs (averages of 6–8 traces; 1 min interval between traces) with the first pair being induced by stimulating first the SR and then the SO after a 200 ms delay; and then the second pair being induced by repeating the stimulation in reversed order. (C) Compiled results showing the mean amplitude (±S.E.M., $n = 11$ neurons) of an IPSC when it was elicited as the second of a pair, normalized for its amplitude when elicited first. When SO stimulation preceded SR stimulation, the SR-elicited IPSC (SR-IPSC) was significantly smaller than when the stimulation order was reversed. No decrement was seen in SR-IPSC if MLA was used to block α7-nAChRs, or the slices were taken from α7-nAChR knockout mice, or the recording pipette was used to infuse BAPTA or the kinase inhibitors KN93 and U0126 into the postsynaptic neuron. Stimulation order produced no decrement in SO-elicited IPSCs (SO-IPSC) under any conditions (reconfigured from Ref. [34]).

can infer the effects of cholinergic input on the IPSC (Fig. 2A). Both stimuli elicited IPSCs in the SR interneuron, but the amplitude of the SR-elicited IPSC was smaller when it was preceded by the SO-elicited IPSC (Fig. 2B). The reverse was not true: the size of the SO-elicited IPSC was independent of order, consistent with the view that only SO stimulation recruited cholinergic responses that could modulate IPSCs. The effect of SO stimulation on SR-elicited IPSCs was blocked by MLA and was absent in hippocampus from α7-nAChR knockout mice, demonstrating the role of α7-nAChRs. No effect of SO stimulation was observed on paired-pulse ratio measured for SR-elicited IPSCs, arguing against a presynaptic effect [34]. Further evidence for a postsynaptic effect of α7-nAChRs came from experiments showing that the ability of SO stimulation to reduce SR-elicited IPSCs was prevented by infusing the neuron either with BAPTA or with the kinase blockers KN-93 and U0126 (Fig. 2C). These results mirror those reported for isolated CG neurons and indicate that α7-nAChR activation can acutely diminish $GABA_A$ responses. Additional experiments examining the recovery of GABA responses after repeated SR stimulations ± MLA indicated that endogenous activation of α7-nAChRs in the slices produces a steady-state regulation of GABAergic responses [34]. The results demonstrate a rapid and reversible inhibition of $GABA_A$ receptors mediated by α7-nAChRs on the postsynaptic cell.

4. Nicotinic timing of GABAergic conversion during development

A different and potentially more profound interaction between nicotinic and GABAergic transmission that we examined concerns the interdependency of these two forms of signaling during development. GABAergic signaling is excitatory during late embryogenesis and early postnatal life, due to an immature chloride gradient [35–37]. This excitatory phase is essential for normal development and integration of neurons into circuits [36,38,39]. We find that endogenous nicotinic activity determines when GABAergic signaling converts from excitation to inhibition during development, and that it does so by changing the chloride gradient across the membrane.

We first tested this hypothesis on chick CG neurons because they express high levels of nicotinic and $GABA_A$ receptors in close juxtaposition on the cell soma and receive GABAergic, as well as cholinergic, input in ovo [40]. Manipulation of nicotinic activity was achieved in ovo by applying either MLA or αBgt to block α7-nAChRs and dihydro-β-erythroidine (DHβE) to block heteromeric nAChRs on the neurons for multiple days. GABA responses were assessed initially by dissociating the neurons from freshly dissected ganglia, loading the cells with the calcium indicator dye Fluo-3-AM, and then quantifying the fluorescent response to GABA. When the chloride gradient is immature, activation of $GABA_A$ receptors depolarizes the membrane, activates VGCCs, and permits calcium influx which generates phasic fluorescence. By this criterion the chloride gradient matures between embryonic day (E) 9 and E14 (Fig. 3A–C). Strikingly, pharmacological blockade of nicotinic receptors in ovo between E8 and E14 caused retention of the excitatory GABAergic response at E14 (Fig. 3D). Patch-clamp recording with the perforated-patch technique to preserve the endogenous chloride gradient confirmed that nicotinic blockade caused the neurons to retain a depolarizing chloride gradient (Fig. 3E–G). Consistent with this, Western blots indicated that the treated neurons retained abnormally high levels of the chloride transporter, NKCC1, which is responsible for the immature chloride gradient causing depolarizing GABAergic responses (Fig. 3H and I).

Analysis of freshly dissociated chick spinal cord neurons revealed a similar phenomenon. Loading with Fluo-3-AM and challenging with GABA plus glycine showed that the GABA/glycine response usually converted from excitation to inhibition between E6 and E9. When treated with MLA and DHβE starting at E3, many of the neurons retained a depolarizing GABA response at E9 [40].

The availability of α7-nAChR knockout mice enabled us to test directly the role of α7-nAChRs in converting the GABAergic signal in the hippocampus. Preparing freshly dissociated hippocampal cells, loading them with Fluo-3-AM, and challenging with GABA demonstrated that the GABA response converted from excitation at P6 to inhibition in most cells by P13. A significantly higher fraction of cells from α7-nAChR knockouts retained a depolarizing response at P13. Moreover, Western blot analysis indicated that hippocampal tissue from the α7-nAChR knockouts retained an immature pattern of chloride transporters, namely high levels of NKCC1 and low levels of KCC2, compared to wildtype tissue of the same age [40]. These results showed that nicotinic signaling helps drive conversion of GABAergic signaling during development in the mammalian hippocampus as it does in the avian autonomic and central nervous systems.

5. Multi-layered nicotinic/GABAergic interactions shaping development

Changing the duration of the GABAergic excitatory phase during development should have significant effects on neuronal maturation and innervation. Less clear is whether the inhibitory phase might also offer developmental instructions, though of a different nature. We tested this idea in CG neurons where nicotinic excitation combined with inhibition can alter gene expression. Thus, nicotinic transmission can activate the transcription factor CREB and change gene expression if, and only if, the membrane potential is kept sufficiently negative such that VGCCs are not routinely activated [15]. Indeed, tests on freshly dissociated E14 CG neurons demonstrated that co-application of GABA, along with nicotine, does enable nicotine to activate CREB when it would not do so alone.

Additional tests were performed in cell culture under conditions where CG neurons retain a depolarizing chloride gradient. Transfecting the neurons with a construct expressing KCC2 inverted the chloride gradient, making GABA inhibitory. Under those conditions, application of GABA-induced unipolarity in the neurons (singe neurite, as in vivo) and restricted innervation of the cell if, and only if,

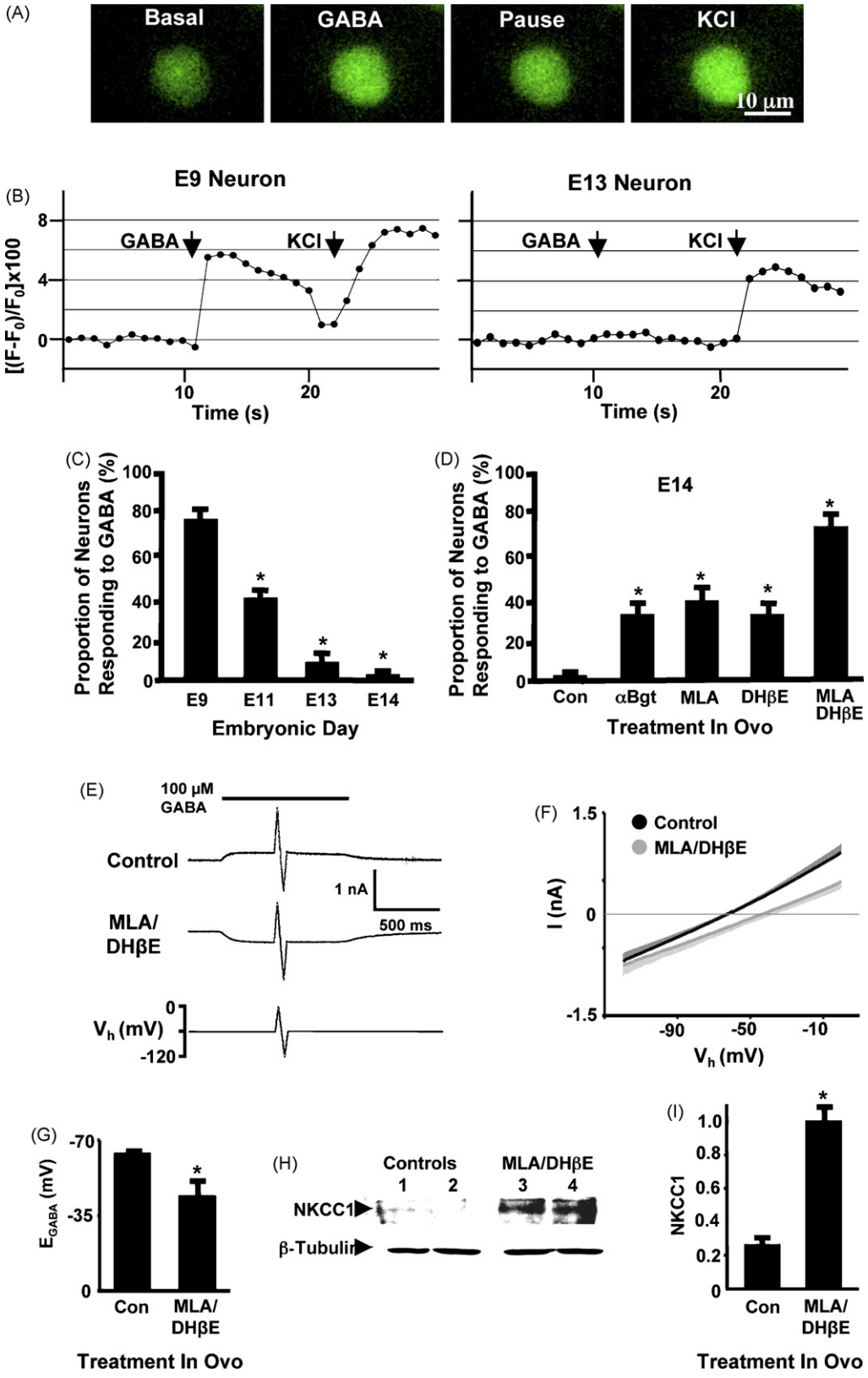

Fig. 3 – Nicotinic blockade in ovo extends the period of GABAergic excitation in CG neurons by delaying maturation of the chloride gradient. (A) E14 CG neurons containing calcium fluor imaged before (Basal) and immediately after applying GABA (GABA), waiting 10 s (Pause) and then stimulating with KCl (KCl). Scale bar: 10 μm. (B) Fluorescence responses of an E9 and an E13 neuron. (C) GABA-induced calcium fluorescence is largely lost in CG neurons between E9 and E14. (D) In ovo application of nicotinic antagonists at E8 caused the neurons to retain a GABA-induced calcium fluorescence response at E14. Con, sham-operated control. Values represent the mean ± S.E.M. (*n* = 18 cultures from six experiments; 200–350

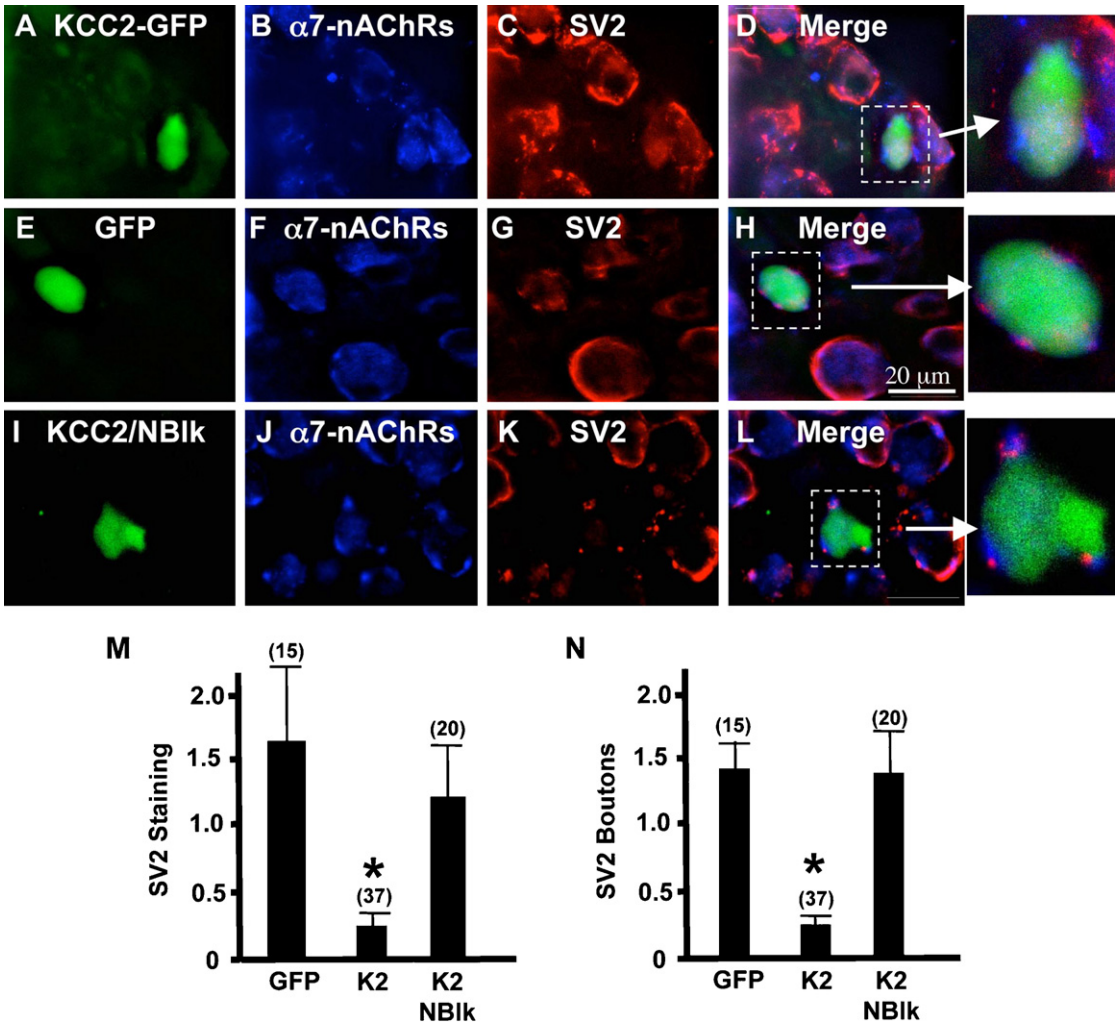

Fig. 4 – Early expression of KCC2 in ovo reduces innervation of choroid neurons unless nicotinic activity is blocked. Neurons electroporated at E1.5 in ovo with KCC2-GFP (A–D), GFP (E–H), or KCC2-GFP and treated with MLA/DHβE (I–L) were imaged at E14 in CG sections after staining for α7-nAChRs and SV2, and merging the images (horizontal rows). Arrows indicate blow-ups. The total amount of SV2 puncta staining (M) and the number of SV2 puncta (N) on the electroporated cell were normalized to that found on adjacent untransfected cells of equivalent size. Scale bar: 20 μm. Values represent mean ± S.E.M. (n = number of electroporated neurons). $^{*}p < 0.05$ and 0.001 for M and N, respectively, by ANOVA (from Ref. [40]).

spontaneous nicotinic activity proceeded without blockade. If, in contrast, nicotinic antagonists where included, then the KCC2 transfection still induced a mature chloride gradient as reflected by the inability of GABA to induce calcium-dependent Fluo-3 fluorescence, but the neurons remained multipolar and continued to be innervated even when chronically treated with GABA. In short, GABAergic inhibition by itself had no effect on these developmental parameters unless nicotinic excitation was allowed to continue [40].

These findings were tested further by electroporating a construct encoding KCC2 fused to green fluorescent protein (GFP) into chick embryos at E1.5, and allowing the embryos to develop to E14. Analysis of ganglion slices at E14 showed that a number of neurons were expressing KCC2-GFP. Focusing on the choroid neurons, which comprise half of the neurons in the ganglion, we found that early expression of KCC2 reduces the number of presynaptic boutons formed on the soma and the amount of immunostaining for the presynaptic antigen

neurons/condition). $^{*}p \leq 0.001$ vs. E9 for C, and vs. Con for D by ANOVA. (E) Perforated patch-clamp recordings from E14 neurons from a control (top) and MLA/DHβE-treated (middle) embryo in response to applied voltage (V_h; bottom). (F) Mean GABA-induced current as a function of V_h in neurons from control (dark line) and MLA/DHβE-treated (light line) embryos. Line widths indicate S.E.M. (n = 7). (G) Mean interpolated GABA reversal potential for neurons from control and MLA/DHβE-treated embryos. $^{*}p < 0.05$, unpaired Student's t-test. (H) Western blots of E14 CGs from control and MLA/DHβE-treated embryos, probed with anti-NKCC1 and anti-β-tubulin (type III) antibodies. (I) Quantification of NKCC1 on Western blots (n = 6 lanes/condition; 10 CGs/lane) (from Ref. [40]).

SV2 (Fig. 4A–D). The neurons did still produce surface clusters of α7-nAChRs as did control neurons. Electroporating with a GFP construct as a negative control produced no effect (Fig. 4E–H). Importantly, the KCC2 effect depended critically on nicotinic activity. If the nicotinic blockers MLA and DHβE were present from E3 onwards, neurons expressing KCC2-GFP were indistinguishable at E14 from GFP-expressing controls (Fig. 4I–N). These results indicate that the appearance of inhibitory GABAergic signaling not only terminates the developmental influence of the excitatory phase but also that it can direct development along new lines if the GABAergic inhibition is coincident with nicotinic excitation in the case of CG neurons.

6. Concluding remarks

Spontaneous waves of endogenous nicotinic activity are common in the developing nervous system. The experiments reviewed here identify one function of nicotinic activity as being the conversion of GABAergic signaling from excitation to inhibition during development. The GABAergic conversion is important because it terminates the developmental impact of the excitatory phase and introduces a new phase in which nicotinic excitation, coupled with GABAergic inhibition, provides different developmental instructions. This reveals a multi-tiered activity-dependent strategy controlling neuronal development. The proximity of α7-nAChRs and $GABA_A$ receptors on developing neurons also enables nicotinic input to have local effects on GABAergic signaling; the developmental consequences of local effects have yet to be explored.

Several questions immediately arise. Does nicotinic activity act directly or indirectly to mediate these widely expressed changes in GABAergic signaling? Many neurons may have sufficient nAChR levels to be under immediate control of nicotinic input. Alternatively, the high levels of nAChRs found on interneurons may position them to relay nicotinic excitation in the form of widely distributed GABAergic excitation that ultimately changes the chloride gradient in recipient cells. A second question concerns the generality of the principles. Do newborn neurons in adult tissue display the same dependence on nicotinic stimulation for GABAergic maturation? Such neurons develop in an entirely different environment from that encountered by neurons in late embryonic and early postnatal stages. A third question concerns the multiplicity of nicotinic actions during development. Might other features of the developing nervous system directly depend on nicotinic activity for efficient execution? More questions than answers emerge—a challenge for the future.

Acknowledgements

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