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Endogenous neurotransmitter activates *N*-methyl-D-aspartate receptors on differentiating neurons in embryonic cortex

(glutamate receptor/neuronal differentiation/cortical development)

MARK G. BLANTON*, JOSEPH J. LO TURCO, AND ARNOLD R. KRIEGSTEIN

Department of Neurology and Neurological Sciences, Stanford University Medical Center, Stanford, CA 94305

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ABSTRACT **Before synapses form in embryonic turtle cerebral cortex, an endogenous neurotransmitter activates *N*-methyl-D-aspartate (NMDA) channels on neurons in the cortical plate. Throughout cortical development, these channels exhibit voltage-dependent Mg^{2+} blockade and are antagonized by D-2-amino-5-phosphonovaleric acid, a selective NMDA receptor antagonist. The activation *in situ* of these nonsynaptic NMDA channels demonstrates a potential physiological substrate for control of early neuronal differentiation.**

N-Methyl-D-aspartate (NMDA) receptors, in addition to their well-recognized role in synaptic plasticity (1–4), may play an equally important role in the control of neuronal growth and differentiation in the central nervous system (5, 6). Results from experiments using neurons growing in culture suggest that NMDA receptor activation can promote neuronal survival (7, 8) and, by regulating intracellular Ca^{2+} levels in growth cones during neurite outgrowth (9, 10) can influence neuronal form (7, 8). If similar mechanisms are to operate during normal development, then NMDA receptors on young neurons must be activated by endogenously released neurotransmitter. The recent development of techniques for whole-cell voltage-clamp recording in intact embryonic brain (11) made it possible to test directly whether NMDA receptors are activated during early neuronal differentiation.

The early anatomical differentiation of the cerebral cortex proceeds along common lines in all higher vertebrate species (12). Neurons are generated in a ventricular zone, migrate radially toward the pia, and collect in a layer to form the cortical plate. We chose turtles for our physiological studies because the intact embryonic turtle forebrain can be maintained *in vitro*, and whole-cell recordings can be obtained from cortical neurons (11), allowing analysis of the development and properties of functional NMDA receptors *in situ*.

MATERIALS AND METHODS

Fertilized eggs of red-eared turtles (*Pseudemys scripta*, from Tangi Turtles, Ponchatoula, LA) were kept moist in an incubator at 30°C; embryos selected for use were staged according to the morphological criteria of Yntema (13). Embryonic turtles were removed from eggs and anesthetized with hypothermia according to Stanford University guidelines for the care and use of animals. Incisions were made in brain hemispheres at rostral and caudal levels and the midline, allowing the cortex to be flattened, ventricular side up.

The preparation, secured on the stage of an upright microscope, was superfused with Ringer's solution (1.5 ml/min at 22–24°C) containing 96.5 mM NaCl, 2.6 mM KCl, 2 mM $CaCl_2$, 31.5 mM $NaHCO_3$, and 10 mM dextrose. Bathing solution also contained 0.3–0.5 μM tetrodotoxin, 5–10 μM bicuculline methiodide, and in most experiments, the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at 4 μM. NMDA receptor antagonists D-2-amino-5-phosphonovaleric acid (D-APV; ref. 14) and Mg^{2+} were added to the bathing solution at 50–250 μM and 2 mM, respectively.

Patch electrodes (5 MΩ) contained 122 mM cesium gluconate, 0.9 mM $CaCl_2$, 0.9 mM $MgCl_2$, 9 mM Hepes, 10 mM EGTA, and 1% biocytin (pH 7.3). These solutions were chosen to minimize voltage- and ligand-gated currents other than those mediated by NMDA receptors. Whole-cell recordings were obtained from cells in intact cortex. Neurons were identified by resting potentials of −50 to −70 mV in current-clamp, high-input impedance (>300 MΩ), Ca^{2+}-mediated action potentials and by morphology after processing for biocytin. Data from whole-cell recordings in voltage clamp, obtained by using a List EP7 patch-clamp amplifier, were filtered at 3 kHz and stored on magnetic tape for off-line analysis. Single-channel data were filtered at 1–1.5 kHz [8-pole Bessel, −3 decibels (dB)] and analyzed by using pCLAMP software (Axon Instruments, Burlingame, CA). Current-level changes briefer than 0.75 msec were ignored in the analysis.

Fluctuation analysis was performed with the SPAN program (provided by J. Dempster). Difference spectra for channels activated by endogenous agonist were calculated by subtraction of background spectra observed in the presence of antagonists from spectra of spontaneous channel-mediated noise. Spectra were fitted by a Lorentzian function, $S_f = S_o \cdot 1/[1 + (f/f_c)^2]$, or by a sum of two Lorentzian functions in which S_f is the power density at frequency f and f_c is the frequency at which S_f is half-maximal ($S_o/2$). Mean channel open time (τ) was determined from the relation: $\tau = 1/2 \cdot \pi \cdot f_c$. Difference spectra for focal glutamate applications were generated by subtracting background spectra obtained prior to agonist application from channel spectra obtained during and following glutamate application.

RESULTS

In embryonic turtles, the first neurons to arrive in the cortical plate begin to elaborate neurites at developmental stage 15 (15). At this early stage of development, when glutamate is present but synapses have not yet formed (16)†, spontaneous channel currents with characteristic properties of NMDA

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Abbreviations: NMDA, *N*-methyl-D-aspartate; D-APV, D-2-amino-5-phosphonovaleric acid.

*To whom reprint requests should be addressed.

†Glutamate was detected in cortical plate neurons at stage 15 using a polyclonal antisera provided by O. Ottersen and J. Storm-Mathisen (M.G.B. and A.R.K., unpublished data). Neither spontaneous nor evoked synaptic currents have been detected prior to stage 18 in embryonic turtles (unpublished data), and similarly, synapses have not been detected at the ultrastructural level until after stage 18.

channels were detected in whole-cell recordings from cortical plate neurons (n = 15; Fig. 1). The channels had an extrapolated reversal potential near 0 mV, the equilibrium potential for cations, as would be expected for NMDA channels. The single-channel conductance was 46 ± 3 pS (mean ± sample SD; n = 4), resembling previously reported values for NMDA receptors (17–19).

The open-time distributions of channel events at stages 15–17 were fitted with two exponentials to give mean open-time components of 1.1 ± 0.08 msec and 4.39 ± 0.21 msec, with the faster component predominant (n = 4; Fig. 1*B*). The shorter open time is similar to reported short components of NMDA receptor open times (17, 19, 20); the longer open time resembles values obtained from whole-cell NMDA receptor currents in cultured neurons (17, 18, 21, 22). To compare channel open times between young neurons with few active channels and older neurons with multiple simultaneously active channels, we also used an alternative method, fluctuation analysis of channel-mediated current (23). In young neurons (stages 15–17), this method indicated a mean open time of 2.80 ± 0.59 msec (n = 7).

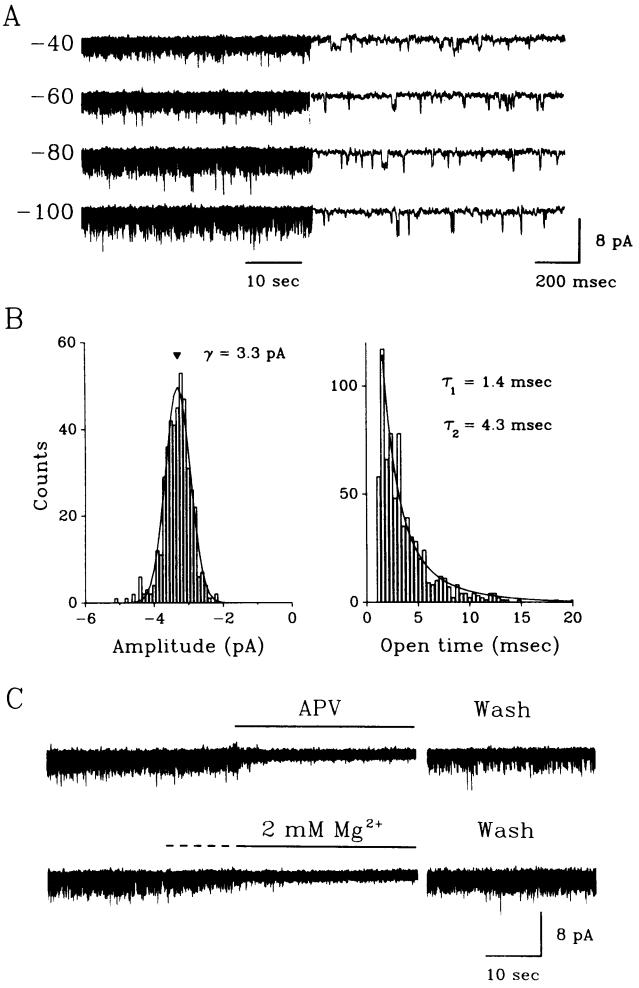

FIG. 1. NMDA receptor-mediated single-channel events are activated by an endogenous agonist in early differentiating turtle cerebral cortex (stage 15). (*A*) Spontaneous inward currents (downward deflections) are resolvable as channel events on the faster time base to the right. The amplitudes of single-channel currents decrease with depolarization, with an extrapolated reversal near 0 mV. (*B*) The amplitude distribution histogram at a holding potential (V_{hold}) of −70 mV, shown to the left, reveals a mean current of 3.3 pA, corresponding to a channel conductance of 47 pS. The open-time distribution at −70 mV, shown to the right, was fitted with two exponentials, τ = 1.4 and 4.3 msec, with the shorter open time predominant. (*C*) Single-channel events in 0 Mg^{2+} (V_{hold} = −70 mV) were reversibly blocked by the competitive NMDA receptor antagonist D-APV (250 μM) and the channel blocker Mg^{2+} (2 mM); the lower trace was taken from a stage 17 neuron.

In addition to characteristic biophysical properties, one would expect certain pharmacologic properties if the observed channels were NMDA receptor channels. First, channels should be blocked by the competitive NMDA receptor antagonist D-APV. As depicted in Fig. 1*C*, the channels were reversibly blocked by 50–250 μM D-APV (n = 10). Second, at hyperpolarized potentials, Mg^{2+} should occlude current flow through the channels (17, 20, 21). When cells were held at −70 mV, channels were blocked upon addition of 2 mM Mg^{2+} to the recording medium (n = 10; Fig. 1*C*). The channels therefore have the antagonist sensitivity and Mg^{2+}-blockade characteristic of NMDA receptor channels. Since D-APV blocks NMDA receptor current by displacing agonist, we conclude that NMDA receptor-gated channels in differentiating cortical neurons are activated by an endogenous agonist, possibly glutamate.

To determine if exogenous glutamate could mimic the effects of the endogenous neurotransmitter, glutamate was focally applied to young neurons at stages 15–17, with non-NMDA receptors blocked by the antagonist CNQX (Fig. 2). Glutamate evoked a current with a peak amplitude near −20 mV (n = 5) that, like the endogenous current, was attenuated at hyperpolarized potentials in 2 mM Mg^{2+}. In nominally Mg^{2+}-free bathing solution, the attenuation of current at hyperpolarized potentials was largely removed, as expected by relief of the magnesium-dependent blockade of the NMDA channel (not shown). The glutamate-evoked current also was attenuated by D-APV in a dose-dependent way (n = 6). In addition, the single-channel conductance, measured in the period of increased channel activity following glutamate application (Fig. 2), was 50 pS, similar to that of the endogenously activated channels. Although applied glutamate and endogenous agonist activated similar conductances, applied glutamate activated a much greater NMDA receptor-mediated current than the endogenous transmitter, indicating that the baseline endogenous agonist concentration is not sufficient to activate all available NMDA receptors.

The mean open time of channels activated by focal glutamate applications in Mg^{2+}-free conditions at stages 15–17 was determined by using fluctuation analysis (23). Power spectra were fitted by single Lorentzian functions, giving a mean channel open time of 4.67 ± 0.57 msec (n = 9). In a particularly favorable recording, an additional component of 0.9 msec could be resolved, resembling the shorter of the two components obtained from analysis of endogenously activated single channels (Fig. 2*C*). The results show that, while similar in their other properties, channels when activated by focal applications of exogenous glutamate had longer mean open times (4.67 ± 0.57 msec) than when activated by endogenous agonist in immature neurons at the same age (2.80 ± 0.59 msec; $P < 0.0001$, two-tailed t test).

To determine how the spontaneous NMDA receptor-mediated current changes with neuronal maturation, the current was compared between cells differing in radial position (and thus maturation) in the same embryo and was also compared between cells from embryos at different stages of development up to stage 26 (hatching). The use of the intracellular label biocytin in the recording pipettes allowed us to correlate cellular morphology and location with the physiological record (11). In recordings at stages 15–18 from cells in the ventricular zone (n = 5), where cells are least mature and glutamate-like immunoreactivity is lowest (M.G.B. and A.R.K., unpublished data), channel events were less frequent than in the cortical plate (Fig. 3). In occasional cells at stage 18 and all cells at older stages (stages 21–26), currents in cortical plate cells were no longer resolvable as single-channel

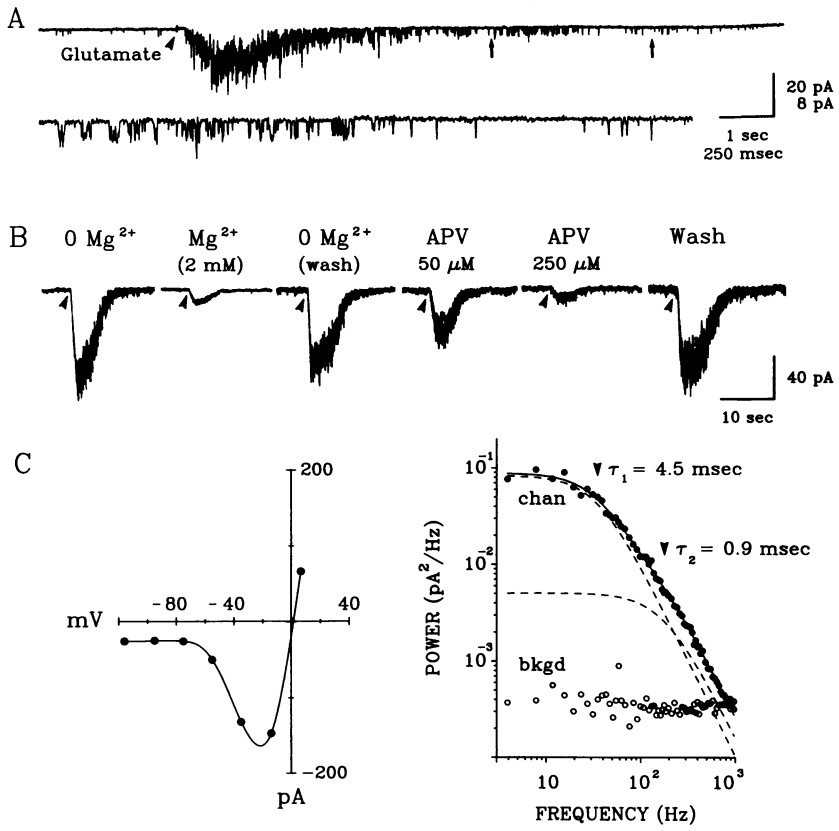

FIG. 2. Focal glutamate application (1 mM) at stage 15 activates currents with properties characteristic of NMDA channels. (*A*) When non-NMDA receptors are blocked by 4 μM CNQX, focal application of glutamate (arrowhead) activates an inward current associated with an increase in current noise (V_{hold} = −82 mV). Glutamate-activated channels, observed in the area between the arrows in the upper trace and enlarged below, have the same conductance (approximately 50 pS) as channels activated by endogenously released agonist. (*B*) Macroscopic glutamate-evoked currents at −70 mV are reversibly attenuated by Mg^{2+} and by D-APV (50 and 250 μM) in the presence of 4 μM CNQX. (*C Left*) Plot of glutamate-evoked currents as a function of membrane potential in the presence of 2 mM Mg^{2+}, which illustrates the characteristic voltage-dependent block of NMDA receptor current. (*C Right*) Power spectrum from the glutamate-evoked currents (chan) of the cell shown in *A*, fit by a sum (———) of two Lorentzian functions (– – –), and background current (bkgd) of the same cell. The corner frequencies (arrowheads) indicate open-time components (τ) of 4.5 msec (f_{c1} = 35.2 Hz) and 0.9 msec (f_{c2} = 181 Hz) (10).

events but appeared as sustained background fluctuations. By stage 26, a sustained inward current of 13.7 ± 4.5 pA (n = 6) was present at −70 mV and could be attenuated by NMDA receptor antagonists. Like the single-channel currents, the sustained current was blocked reversibly by D-APV (n = 6) and at −70 mV by 2 mM Mg^{2+} (n = 14; Fig. 3*B*). Fluctuation analysis of these currents gave open times of 5.05 ± 1.43 msec (n = 3) at stage 21 and 6.19 ± 1.03 msec (n = 7) at stage 26, consistent with reported open times of NMDA receptor channels (17, 18, 21, 22, 24). These observations indicate that as neurons grow and differentiate, they acquire an increasing background current mediated by NMDA receptors.

DISCUSSION

Application of whole-cell recording in intact cortex has allowed analysis of neurotransmitter receptor function in

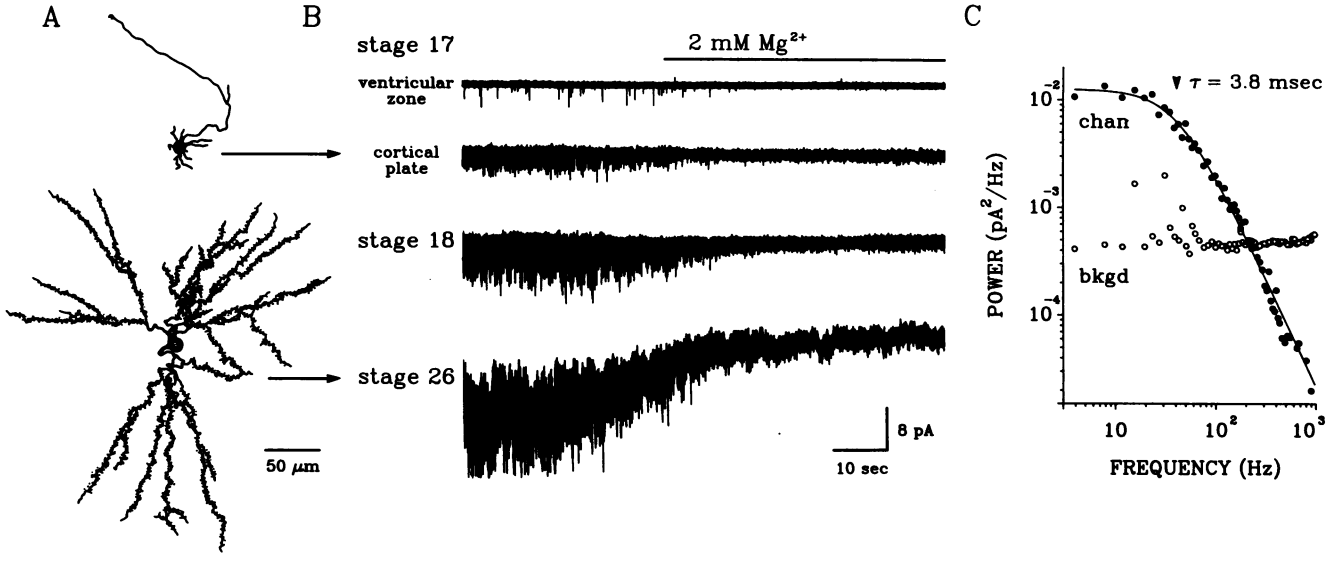

FIG. 3. Spontaneous current events increase in frequency as neurons develop and elaborate neurites. (*A*) Camera lucida drawings of biocytin-labeled pyramidal cells (in the horizontal plane) illustrate the tremendous elaboration of dendritic complexity during embryonic life from stages 17–26. Arrows indicate the currents in *B* that were recorded from the neurons illustrated in *A*. (*B*) At stage 17, the frequency of Mg^{2+}-sensitive channel events was low in cells in the ventricular zone and was higher in cells in the cortical plate, where cells are more mature and have begun to differentiate. Cortical plate neurons in older embryos show a further increase in the frequency of current events, and the current becomes a sustained baseline noise (stage 18). In later embryonic stages (stage 26), the NMDA receptor events are sufficiently frequent to produce a continuous inward current revealed by Mg^{2+} blockade. (*C*) Power spectrum from the stage 18 pyramidal cell in *B* when fitted with a single Lorentzian function, gives an open time (τ) for the channel (chan) of 3.84 ms (channel in 0 Mg^{2+}; background in 2 mM Mg^{2+}).

developing vertebrate neurons. We have shown here that an endogenous neurotransmitter is present and can activate NMDA receptors on differentiating neurons at the earliest stages of cortical morphogenesis. The presence of functional NMDA receptors *in situ* provides crucial support for the idea, based on studies in culture (5–8, 9, 10, 25), that neurotransmitters *in vivo* may influence early cortical development.

The only observed parameter of NMDA receptor function that changed in development was mean open time of endogenously activated channels, which increased from 2.80 ± 0.59 msec at stages 15–17 to 6.19 ± 1.03 msec at stage 26 ($P < 0.0001$, two-tailed t test). This trend could theoretically result from developmental changes in the type or concentration of agonist (26), changes in receptor subunits (27), or changes in channel regulation by intracellular or extracellular modulators (28). Alternatively, different open times could be an artifact of the filtering of higher frequency noise components by cell resistance and capacitance in larger, more mature cells, causing an underestimate of fast channel events in older cells (19, 22). However, filtering could not explain the lack of longer spontaneous events in young cells, and longer open time events were observed in young neurons after focal glutamate applications. Therefore, the predominance of short open times in young neurons could be explained, at least in part, by low agonist concentration in the extracellular space, which has been shown to produce brief open states for the γ-aminobutyric acid type A receptor (26) and possibly the NMDA receptor (19).

We have found that a characteristic feature of NMDA receptor current—its voltage-dependent blockade by Mg^{2+} (17, 20, 21)—was present in all neurons throughout cortical development. Previous studies have demonstrated that voltage-dependent receptor blockade was present in embryonic spinal cord neurons in culture (29). In contrast, NMDA responses of immature hippocampal neurons were reported to have less consistent voltage dependence than responses of mature neurons (30). The Mg^{2+}-dependence of the channels is important because it determines the conditions under which current can flow. When embryonic cortical neurons are at resting membrane potential, glutamate binding to the NMDA receptor would be expected to produce little current because of Mg^{2+} blockade of the channel. However, when a neuron is depolarized, Mg^{2+} is removed from NMDA channels and current will flow (see Fig. 2*C*). Prior to synapse formation, the activity of voltage-dependent and other ligand-gated channels that regulate membrane potential can interact to modulate the amount of this current and as a result can affect neuronal survival (5, 6), neurite growth and morphological differentiation (7, 8, 25), and gene expression (31).

The NMDA receptor-mediated current is present throughout development (this study) and into adulthood (24). It has been proposed that glutamate-mediated currents exert a spectrum of effects on neuronal form and function throughout life (25). Such effects range from enhancement of neurite outgrowth and synaptic plasticity to retraction of processes, neuronal injury, and death. Long-term potentiation, a form of synaptic plasticity related to learning, requires activation of NMDA receptors (3, 4) and may involve morphological changes in synapses and dendritic spines (32, 33). In contrast, excessive NMDA receptor activation can cause retraction of neuronal processes and cell death (34) and could play a role in nervous system disorders like Alzheimer disease (35) and temporal lobe epilepsy (36), in which some neurons appear to lose dendritic complexity and degenerate. The presence of sustained glutamate-activated current observed in developing brain prior to synapse formation and in mature brain may indicate a unifying mechanism for establishing and regulating neural circuitry throughout life.

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