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Kainate Receptors Expressed by a Subpopulation of Developing Nociceptors Rapidly Switch from High to Low Ca^{2+} Permeability

C. Justin Lee,[1] Haeyoung Kong,[4] M. Chiara Manzini,[2,3] Cristóvão Albuquerque,[1] Moses V. Chao,[4] and Amy B. MacDermott[1,3]

[1]Department of Physiology and Cellular Biophysics, [2]Department of Pathology, and the [3]Center for Neurobiology and Behavior, Columbia University, New York, New York 10032, and [4]Molecular Neurobiology Program, Skirball Institute, New York University Medical Center, New York, New York 10016

Dorsal root ganglion (DRG) neurons first express kainate receptor subunits, predominantly GluR5, during embryonic development. In the DRG and throughout the nervous system, substantial editing of GluR5 mRNA occurs with developmental maturation (Bernard et al., 1999). The accompanying change in Ca^{2+} permeability of functional kainate receptors that is the predicted outcome of this developmental regulation of mRNA editing has not been investigated. Here we report that kainate receptors on DRG neurons from late embryonic and newborn rats are predominantly Ca^{2+} permeable but then become fully Ca^{2+} impermeable later in the first postnatal week. Using multiple markers for nociceptor subpopulations, we show that this switch in Ca^{2+} permeability is not caused by the appearance of a new subpopulation of nociceptors with different receptor properties. Instead, the change in Ca^{2+} permeability matches the time course of post-transcriptional RNA editing of GluR5 at the Q/R site within the pore of the channel, indicating that the change is probably caused by developmentally regulated RNA editing. We also report that, on the basis of the strong correlation of receptor expression with expression of the surface markers LA4, isolectin B4, and LD2, kainate receptors are present on C-fiber-type neurons projecting to lamina II of spinal cord dorsal horn. These results raise the possibility that kainate receptors in their Ca^{2+}-permeable form serve a developmental role in synapse formation between this population of C-fibers and their targets in the spinal cord dorsal horn. Thereafter, the receptors may serve a new function that does not require Ca^{2+} permeability.

Key words: dorsal root ganglion; nociceptor; kainate receptor; calcium permeability; capsaicin; LD2; LA4; IB4; lamina II; C-fiber

Kainate receptors, one of the three families of ionotropic glutamate receptors, are expressed by neurons in both the peripheral nervous system and the CNS. The receptors are composed of homomeric and heteromeric configurations of five cloned subunits: GluR5–7 and KA1 and KA2 (for review, see Chittajallu et al., 1999). Post-transcriptional mRNA editing of the GluR5 and GluR6 subunits results in replacement of a glutamine (Q) with an arginine (R) at a site in the channel pore referred to as the Q/R site (Sommer et al., 1991). This amino acid replacement strongly reduces Ca^{2+} permeability (Burnashev et al., 1995) and single-channel conductance of the kainate receptor in heterologous expression systems (Swanson et al., 1996). Editing of GluR5 and GluR6 mRNA subunits begins during late embryonic development throughout the nervous system (Bernard and Khrestchatisky, 1994; Paschen et al., 1995; Bernard et al., 1999). The functional consequences of this change for some channel properties have been investigated (Ruano et al., 1995; Pemberton et al., 1998; Smith et al., 1999), although direct measurement of changes in the Ca^{2+} permeability of kainate receptors during development has not been made.

GluR5 is expressed by a subset of mouse dorsal root ganglion (DRG) neurons early in embryonic development [embryonic day 12 (E12)] (Bettler et al., 1990), suggesting a developmental role for kainate receptors. Bernard et al. (1999) reported that in rat DRGs, the GluR5 receptor subunits change from being mostly unedited at E17 to being partially edited in the adult. These data suggest that there is a dynamic regulation of GluR5 editing during the critical period of late embryonic and early postnatal ages when robust changes in synaptic connectivity are occurring. However, the precise time course of changes in GluR5 editing in DRG neurons remains undetermined. Furthermore, the impact of subunit editing on functional channel properties such as Ca^{2+} permeability must be measured directly because receptor subunit composition as well as subunit editing will determine the relative number of Ca^{2+}-permeable kainate receptors. Therefore, we have directly measured the degree of editing of GluR5 mRNA and the relative Ca^{2+} permeability of kainate receptors expressed by rat DRG neurons at different ages during early development.

One complication with studies of DRG neuronal properties is the heterogeneity of the neurons in the ganglia. Within DRG neuronal subpopulations, kainate receptors are expressed primarily by small-diameter neurons, presumably nociceptors (Huettner, 1990). However, it is clear that by any measure there are multiple subpopulations of nociceptors within the DRG. We have used the carbohydrate surface markers developed by Dodd and Jessell (1985), expression of the peptide substance P, and expression of the noxious heat- and capsaicin-sensitive vanilloid receptor VR1

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Correspondence should be addressed to Dr. Changjoon Justin Lee, Department of Physiology and Cellular Biophysics, Columbia University, 630 West 168th Street BB1106, New York, NY 10032. E-mail: cjl2@columbia.edu.

(Caterina et al., 1997, 2000) to define subpopulations of nociceptors. We have investigated the expression of kainate receptors by subpopulations of DRG neurons and the developmental changes in Ca^{2+} permeability that kainate receptors undergo.

MATERIALS AND METHODS

Acute DRG preparation

Postnatal day 0 (P0) to P8 rats were deeply anesthetized with isoflurane and decapitated, and then cervical, thoracic, and lumbar DRGs were dissected out. To obtain E18 DRG neurons, a pregnant rat was anesthetized with CO_2 and killed. Several embryos were removed and decapitated, and then cervical, thoracic, and lumbar DRGs were dissected out. Isolated DRGs were exposed for 10–20 min at 37°C to 1 mg/ml trypsin (type III; Sigma, St. Louis, MO) dissolved in the external bath solution without $CaCl_2$ and $MgCl_2$. Next, the trypsin was inactivated by addition of an equal volume of 1 mg/ml trypsin inhibitor (type II-O; Sigma) dissolved in the external bath solution with $CaCl_2$ and $MgCl_2$. DRGs were mechanically dissociated with fire-polished glass pipettes. Cells were washed twice by centrifuging and replacing the supernatant with fresh external bath solution. Cells were then plated on poly-D-lysine-coated glass coverslips and placed in an incubator for 1–8 hr. DRG neurons could be easily identified on the basis of their round morphology and large somal size (15–40 μm).

Simultaneous electrophysiology and Ca^{2+} imaging

Acutely dissociated DRG neurons were loaded with 5 μM fura-2 AM for 15–20 min at room temperature and washed. Background-subtracted intensity images at two excitation wavelengths (340 and 380 nm) were acquired using an intensified CCD camera and Axon Imaging Workbench 2.1 (Axon Instruments, Foster City, CA). Detailed methods are given elsewhere (Kyrozis et al., 1995; Gu et al., 1996). Kainate-responsive DRG neurons were initially identified by screening for cells that showed Ca^{2+} responses to 5 sec applications of kainate (100 μM). Then a recording electrode was applied to the kainate-responsive neuron.

All electrophysiological recordings were made using gramicidin perforated-patch electrodes. The pipette solution contained 25 μg/ml gramicidin D, 75 mM Cs_2SO_4, 10 mM NaCl, 0.1 mM $CaCl_2$, and 10 mM HEPES; pH was adjusted to pH 7.1 with CsOH, and osmolarity was adjusted to 310 mOsm with sucrose. Pipette resistances ranged from 3 to 5 MΩ. It took 20–30 min to achieve acceptable perforation, with final series resistances ranging from 15 to 40 MΩ. Under the perforated-patch configuration, the fura-2 does not leak out from the cell body, allowing us to perform simultaneous recordings of whole-cell currents and Ca^{2+} imaging. Drug solutions were applied to cells by local perfusion through a capillary tube (1.1 mm inner diameter) positioned near the cell of interest. The solution flow was driven by gravity (flow rate, ~1–5 ml/min) and controlled by miniature solenoid valves (The Lee Company, Westbrook, CT). Membrane currents or voltages were recorded using an Axopatch 200B amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 2–5 kHz with pClamp 6 acquisition software (Axon Instruments). The external bath solution contained 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 2 mM $MgCl_2$, and 5.5 mM glucose at pH 7.3 and 325 mOsm. For the recordings that required 0 mM Na^+ and 10 mM Ca^{2+} (0Na/10Ca), 145 mM NaCl was replaced by 155 mM *N*-methyl-D-glutamate and 144 mM HCl, in addition to 10 mM $CaCl_2$.

Agonist-evoked currents and Ca^{2+}-imaging data were analyzed using the Mini Analysis Program (Synaptosoft, www.synaptosoft.com). Current amplitudes were calculated as the difference between the baseline and current amplitude at the end of drug application (5 sec). The area under the curve was calculated by integrating a response trace from onset to offset of a drug application. The peak amplitude of the Ca^{2+} response was determined by subtracting the average baseline from the peak ratio value during a drug application. The relative Ca^{2+} permeability of the kainate receptors on each neuron was estimated by calculating the ratio of peak amplitude of the Ca^{2+} response over the corresponding area under the curve of the current response.

The current–voltage (*I–V*) relationships for domoate-induced currents were obtained by performing 10 sec voltage ramps (−100 to +100 mV) before and during 10 μM domoate application. The two traces were subtracted to obtain the *I–V* relationship. The recordings were done in the presence of external bath solution containing 0.5 μM tetrodotoxin (TTX) and 100 μM $CdCl_2$. For some recordings, 10 mM lidocaine was used to block both voltage-gated sodium channels and Ca^{2+} channels (Gu and MacDermott, 1997). The index of rectification for each *I–V* relationship was calculated by taking the ratio of the slope conductance at +60 and −60 mV from each cell (Herb et al., 1992).

GluR5 RNA-editing assay

DRGs were isolated from embryos or postnatal rat pups at different ages (from E16 to P7). To get enough tissue, multiple embryos were dissected and included for each embryonic run per age. For postnatal and adult animals, the DRGs from only one animal were used for each run per age. At least three runs were used at each age, except for the adult that included only one run.

Reverse transcription-PCR. Total RNA was isolated from DRGs by Dounce homogenization in Trizol reagent (Life Technologies, Gaithersburg, MD) as instructed by the manufacturer. Five micrograms of total RNA were reverse transcribed in the presence of oligo-dT and reverse transcriptase. This cDNA template was used for PCR to amplify the region encoding TM2 of GluR5 by use of the upstream primer 5′-GTTTGTGATTGCGAGGTTCACA-3′ and the downstream primer 5′-CAGGTTGGCCGTGTAGGATGA-3′. Cycle conditions were as follows: 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min.

RNA-editing analysis. The extent of Q/R editing in TM2 of GluR5 was assessed by *Bbv*I digestion of the PCR products amplified from rat DRG. The restriction enzyme *Bbv*I recognizes the sequence GCAGC, which exactly corresponds to the sequence of unedited GluR5 mRNA. The amplified PCR product (233 bp) in the unedited state can be cleaved by *Bbv*I to two fragments of 139 and 94 bp. These products were separated on 10% polyacrylamide–TBE gels and stained with ethidium bromide for visualization. Editing of GluR5 from GCAGC to GCGGC renders the latter DNA fragments refractory to *Bbv*I digestion. Thus, the extent of RNA editing can be quantified by calculating the ratio of cleaved to uncleaved DNA.

Immunocytochemistry of dissociated DRG neurons after Ca^{2+} imaging

Dissociated DRG neurons were plated on coverslips with grids (Bellco Glass, Inc., Vineland, NJ) and loaded with fura-2 AM. Kainate (100 μM) was applied to a field with cells for 5 sec. The criterion for the presence of kainate receptors was that the Ca^{2+} responses were five times the root mean square of the baseline Ca^{2+} signal. If there was a peak above this threshold at the time of drug application, the cell was considered positive for kainate receptor expression. Subsequently, bright-field images were taken of each field, and the location of the field was determined by the etched markings on the grid. After screening, the cells were prepared for immunostaining by being fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min.

Coverslips were first incubated in a blocking solution of 10% normal goat serum in PBS with 0.1% Triton X-100 for 30 min. Primary antibodies were diluted in PBS with 0.1% Triton X-100 and 1% normal goat serum (PBS-TG). Polyclonal rabbit anti-VR1 (gift from Dr. David Julius) and anti-substance P (DiaSorin, Stillwater, MN) were used at 1:5000 dilution. SSEA4 (mouse IgG), LD2 (mouse IgM), and LA4 (mouse IgM) were purchased from Developmental Studies Hybridoma Bank (University of Iowa) and were used at dilutions of 1:1, 1:5, and 1:10, respectively. For the double immunostaining with isolectin B4 (IB4) and LA4, biotinylated–conjugated IB4 (Sigma) was used at 1:100 dilution, and Alexa488–streptavidin (Molecular Probes, Eugene, OR) was used as the secondary at 1:500 dilution. Secondary antibody indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was used for anti-VR1 and anti-substance P at 1:500 dilution. Cy3-conjugated goat anti-mouse IgM (Molecular Probes) was used for LD2 and LA4, diluted 1:500. Cy3-conjugated goat anti-mouse IgG (Molecular Probes) was used for SSEA4 at 1:500 dilution. The secondary antibodies were applied in PBS-TG for 30 min. Sections were mounted in ProLong Antifade (Molecular Probes). The cells were identified after immunostaining using the grids. We were able to relocate >95% of the cells that we recorded with Ca^{2+} imaging.

The immunostaining was observed using a Zeiss Axioscope fluorescent microscope. Each staining was compared with a control that did not include primary antibody. Staining was considered positive if a cell displayed at least five times brighter intensity compared with that of the control staining. Most of the time, cells were examined by at least two independent observers to eliminate bias.

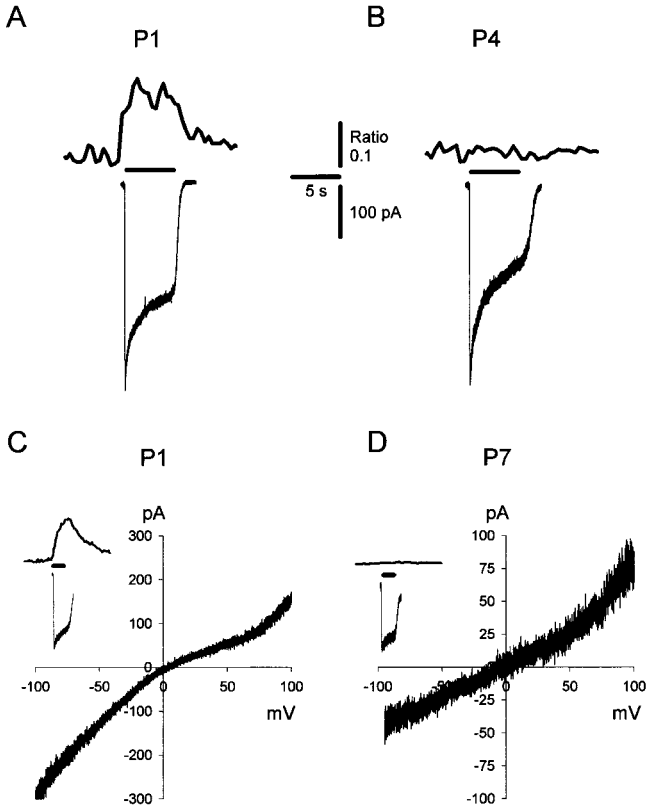

Figure 1. Postnatal DRG neurons express Ca^{2+}-permeable and -impermeable kainate receptors. Acutely dissociated DRG neurons expressing kainate receptors were pre-identified by screening for a change in $[Ca^{2+}]_i$ in response to 100 μM kainate. Neurons were then voltage clamped at −70 mV using the perforated-patched recording configuration. *A*, Kainate-induced inward current (*bottom trace*) is accompanied by a Ca^{2+} increase (*top trace*) in a P1 DRG neuron, indicating activation of Ca^{2+}-permeable kainate receptors. *B*, Kainate-induced current in a P4 DRG neuron shows an amplitude similar to that of the neuron in *A* but with no change in $[Ca^{2+}]_i$, indicating the predominant expression of Ca^{2+}-impermeable kainate receptors. *C*, *I–V* relationship of domoate-induced currents shows a doubly rectifying relation in a P1 DRG neuron different from that shown in *A*. A voltage ramp (−100 to 100 mV) was introduced before and during the 10 μM domoate application. The two resulting *traces* were subtracted to obtain the *I–V* curve as shown. *Inset*, A high degree of Ca^{2+} permeability determined by simultaneous recording of $[Ca^{2+}]_i$ (*top trace*) and current (*bottom trace*) from this neuron ($n = 5$) is shown. The peak amplitudes of the Ca^{2+} response and domoate-induced current are 0.65 and 962 pA. *D*, A similar experiment in a P7 DRG neuron shows an outwardly rectifying *I–V* relationship. *Inset*, The corresponding Ca^{2+} permeability is low ($n = 3$). The peak amplitude of the domoate-induced current is 297 pA. The *horizontal bars* above each current *trace* in *A–D* indicate the duration of drug application.

RESULTS

Ca^{2+} permeability of kainate receptors

The Ca^{2+} permeability of kainate receptors expressed by DRG neurons was assessed using three different approaches. The first approach was to record simultaneously kainate-induced whole-cell currents and Ca^{2+} transients from acutely dissociated postnatal DRG neurons. For this protocol, DRG neurons were screened for sensitivity to kainate using Ca^{2+} imaging. Then a perforated-patch recording electrode was sealed onto each responding neuron, and membrane currents were recorded under voltage clamp. Figure 1*A* shows a recording from a P1 rat DRG neuron. In this example, kainate produced a strong inward current that was accompanied by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In other cases, such as the example shown in Figure 1*B* recorded from a P4 rat DRG neuron, the kainate-evoked inward current was not accompanied by an increase in $[Ca^{2+}]_i$. Because each neuron was voltage clamped and held at −70 mV, there should be minimal contribution from voltage-gated Ca^{2+} channels to the kainate-evoked Ca^{2+} response.

The *I–V* relationship for Ca^{2+}-permeable kainate receptors has been shown to be inwardly rectifying, whereas for Ca^{2+}-impermeable kainate receptors it is not (Burnashev et al., 1995). Therefore, the second approach we used to assess the presence of Ca^{2+}-permeable kainate receptors was a voltage-ramp protocol. Domoate was used as the agonist in this series of experiments because it induces less desensitization of kainate receptors in DRG neurons than does kainate (Huettner, 1990).

Figure 1, *C* and *D*, shows the *I–V* relationships for domoate-evoked currents in DRG neurons from P1 and P7 rat pups, respectively (see Materials and Methods for *I–V* curve generation from voltage ramps). Ca^{2+} permeability was initially tested by recording an $[Ca^{2+}]_i$ response to 10 μM domoate with the membrane voltage clamped at −70 mV as shown in Figure 1, *C* and *D*, *insets*. The ramp protocol was subsequently performed before, during, and after domoate application in the presence of 100 μM $CdCl_2$ to block voltage-gated Ca^{2+} channels and 0.5 μM TTX to block TTX-sensitive voltage-gated Na^+ channel activation during the voltage ramp. In some cases the ramp protocol was performed in the presence of 10 mM lidocaine and 0.5 μM TTX to suppress TTX-sensitive and -resistant sodium channels and voltage-gated Ca^{2+} channels (Gu and MacDermott, 1997). Figure 1*C* shows an *I–V* curve with inward rectification of the domoate-evoked current recorded from a neuron with Ca^{2+}-permeable kainate receptors. In Figure 1*D*, the *I–V* curve recorded from another neuron shows no inward rectification, consistent with the lack of $[Ca^{2+}]_i$ transient associated with the domoate-evoked current. These data demonstrate that the kainate receptors expressed by DRG neurons can be Ca^{2+} permeable or Ca^{2+} impermeable.

Developmental changes in Ca^{2+} permeability of kainate receptors

We tested whether the variable expression of Ca^{2+}-permeable and -impermeable kainate receptors was caused by a developmentally regulated change in Ca^{2+} permeability of the kainate receptor. We recorded from 32 DRG neurons prepared from rat pups at different ages during late embryonic development and the first postnatal week. To confirm that the kainate-induced responses were mediated by kainate receptors and not by AMPA receptors, 26 of 32 neurons were also tested for kainate sensitivity after preapplication of SYM 2081 (Fig. 2). This compound is a selective and strongly desensitizing agonist for kainate receptors (Zhou et al., 1997; Donevan et al., 1998) that we have used as a functional antagonist for kainate receptors on DRG neurons (Lee et al., 1999). In all cells tested in this study, the response to kainate was completely blocked by preapplication of SYM 2081. AMPA receptor-mediated responses to kainate were detected in DRG neurons in the presence of SYM 2081 (C. J. Lee, unpublished observation), but the frequency of occurrence was low (much <1% of neurons tested). Twenty-one of 32 neurons were tested for capsaicin sensitivity (examples in Fig. 2); 71% were positive, indicating that they expressed functional VR1 protein, the receptor for capsaicin and noxious heat.

Kainate was applied in a 0Na/10Ca bath to 5 of the 32 neurons as the third approach to assess Ca^{2+} permeability of kainate

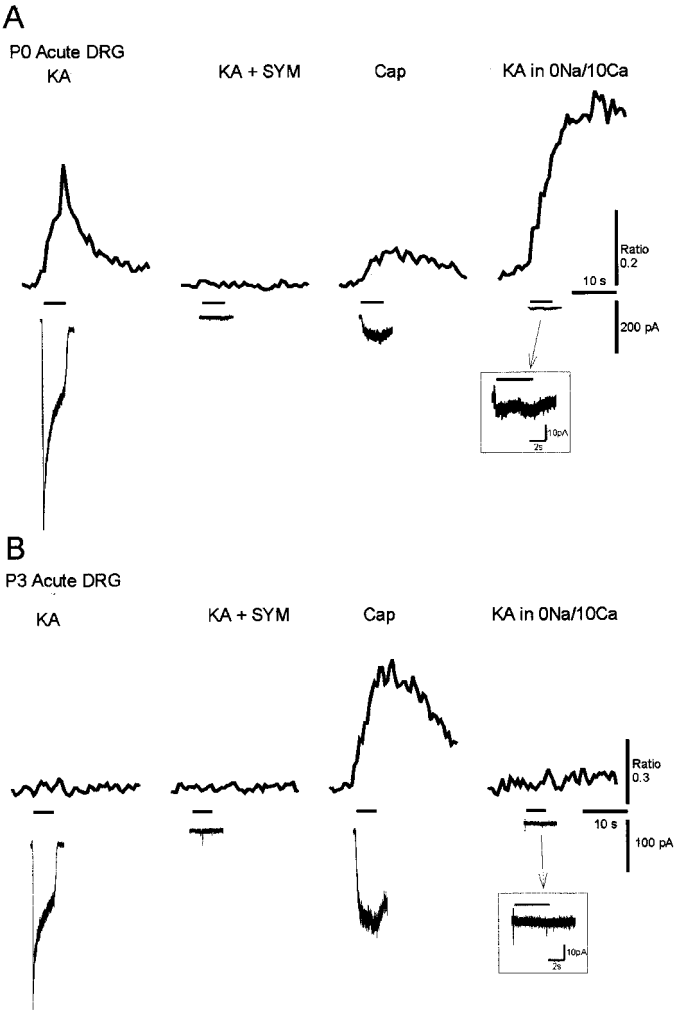

Figure 2. Kainate receptors mediate responses to kainate in >99% of acutely prepared DRG neurons tested in our study. *A*, A pharmacological profile of kainate responses in a P0 DRG neuron is illustrated. Kainate (*KA*)-induced whole-cell currents (*bottom traces*) were accompanied by Ca^{2+} increases (*top traces*). These were blocked by pretreatment with 3 μM SYM 2081 (*KA + SYM*). The neuron also displayed an inward current and Ca^{2+} increase with 1 μM capsaicin (*Cap*). KA induced a small inward current and larger Ca^{2+} increase in the 0Na/10Ca bath solution (*KA in 0NA/10Ca*), providing additional evidence that the kainate receptors are Ca^{2+} permeable ($n = 3$). *B*, A similar series of experiments was done on a P3 DRG neuron. This neuron shows *KA*-induced whole-cell currents without a Ca^{2+} increase, indicating little Ca^{2+} permeability. *KA + SYM* blocked the response, and *Cap* induced an inward current. *KA in 0Na/10Ca* did not show any Ca^{2+} increase, indicating no Ca^{2+} permeability of the receptors ($n = 2$). The *horizontal bars* above each current *trace* indicate the duration of drug application.

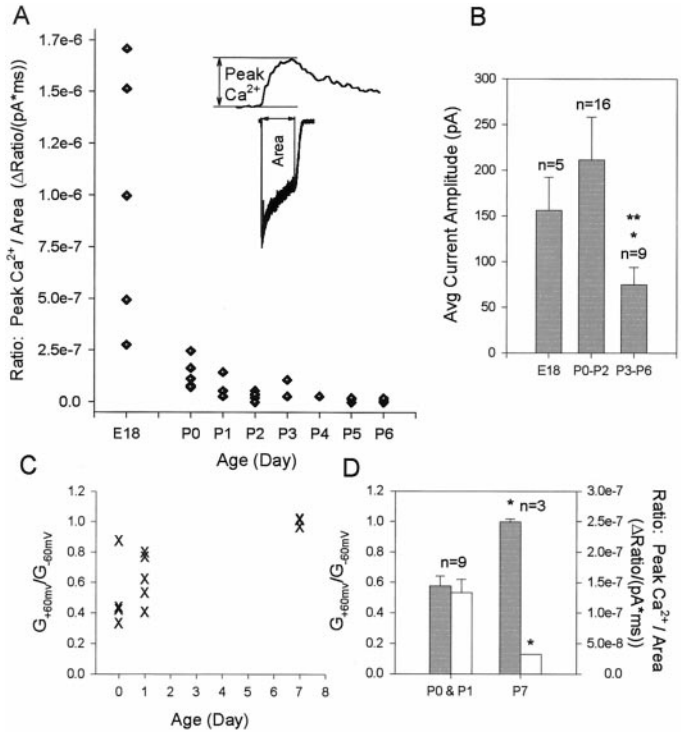

Figure 3. Ca^{2+} permeability of kainate receptors switches off during late embryonic and early postnatal development. *A*, The number of DRG neurons showing high Ca^{2+} permeability decreases with age. Each *symbol* represents a ratio of peak Ca^{2+} response normalized to the *area* under the *curve* of KA-induced current, as demonstrated in the *inset*. Each *symbol* represents one DRG neuron. Day 0 represents the day of birth (total $n = 32$). *B*, The E18 and neonate (P0–P2) animals show a larger current amplitude than do older animals. The average amplitudes of KA-induced currents are binned into three age groups: E18 ($n = 5$), P0–P2 ($n = 16$), and P3–P6 ($n = 9$). *Single* and *double asterisks* indicate significant differences (based on Student's *t* test) between P3–P6 and P0–P2 groups ($p < 0.05$) and between P3–P6 and E18 groups ($p < 0.05$), respectively. There was no significant difference between E18 and P0–P2 groups. *C*, The developmental change in *I–V* relationship is shown. Each *X symbol* represents the rectification index from one DRG neuron. The rectification index is expressed as the ratio of conductance at +60 and −60 mV. *D,* The averages of the rectification indices at different ages are shown (*gray bars*, scale on *left y*-axis) along with the average Ca^{2+} permeabilities, calculated from the domoate-induced currents and Ca^{2+} responses (as in *A*; *white bars*, scale on *right y*-axis). The *single asterisks* indicate significant differences (based on Student's *t* test) between P0–P1 and P7 groups ($p < 0.05$). The error bars indicate the SEM.

receptors. Figure 2*A* shows *traces* from a P0 DRG neuron expressing Ca^{2+}-permeable kainate receptors as indicated by the kainate-induced change in $[Ca^{2+}]_i$. In contrast, data recorded from a P3 neuron shown in Figure 2*B* show no change in $[Ca^{2+}]_i$ associated with kainate current. Neurons with a kainate-induced increase in $[Ca^{2+}]_i$ in normal bath showed a larger change in $[Ca^{2+}]_i$ in 0Na/10Ca bath, indicating that some kainate receptors were indeed Ca^{2+} permeable ($n = 3$; Fig. 2*A*). This increase in $[Ca^{2+}]_i$ in 0Na/10Ca bath was accompanied by a small but substantial inward current as shown in Figure 2*A*, *inset*. Neurons that did not show a kainate-induced elevation in $[Ca^{2+}]_i$ in normal bath under voltage clamp did not show a Ca^{2+} response or an inward current in 0Na/10Ca bath, indicating that essentially all of the kainate receptors were Ca^{2+} impermeable ($n = 2$; Fig. 2*B*).

The change in relative Ca^{2+} permeability of kainate-evoked currents as a function of postnatal age is shown in Figure 3*A*. The relative expression of Ca^{2+}-permeable and -impermeable kainate receptors by individual DRG neurons was determined by simultaneous measurements of kainate-evoked changes in $[Ca^{2+}]_i$ and membrane current. Ca^{2+} permeability was expressed as the ratio of the peak Ca^{2+} response divided by the area under the curve of the corresponding current trace (see Fig. 3*A*, *inset*). High values of this ratio indicate a neuron expressing a higher proportion of kainate receptors that are Ca^{2+} permeable. As shown in Figure 3*A*, a greater proportion of neurons demonstrates high Ca^{2+} permeability at E18 and at approximately the time of birth. With increasing age, however, this permeability decreases. By P4 and older, most of the neurons displayed ratios close to zero, indicating that few or no Ca^{2+}-

permeable kainate receptors contribute to the kainate-evoked currents from those cells. These results indicate that kainate receptors switch from Ca^{2+} permeable to Ca^{2+} impermeable at approximately the time of birth.

The overall kainate-evoked current amplitude decreased over the first postnatal week along with the Ca^{2+} permeability of kainate receptors, although not as dramatically. We grouped the current amplitude data into cells from three age groups: E18, P0–P2, and P3–P6. As shown in Figure 3*B*, neurons from E18 and P0–P2 pups showed significantly larger average current amplitudes than did neurons from P3–P6 pups. This demonstrates that age-dependent changes in Ca^{2+} permeability coincide with a decrease in current amplitude, raising the possibility that Ca^{2+} transients evoked by kainate are simply more difficult to detect from older neurons. However, as demonstrated in Figure 1, *A* and *B*, there were many kainate-evoked currents with similar amplitudes yet with substantial differences in the size of the $[Ca^{2+}]_i$ transients.

If the Ca^{2+} permeability of kainate receptors is changing over the first postnatal week, the average shape of the *I–V* relationship for domoate-evoked currents is also predicted to change over this time. The shapes of the *I–V* relationships were compared by calculating a rectification index that should correlate with the relative number of Ca^{2+}-permeable kainate receptors. We calculated the index of rectification for kainate-evoked currents from P0–P1 and P7 rats, and the results are shown in Figure 3, *C* and *D*. The index of rectification used here is the ratio of slope conductances for domoate-evoked current at +60 and −60 mV. A ratio of <1 indicates inward rectification. The index of rectification values plotted for cells from the P0 and P1 animals are all <1 (Fig. 3*C*). Average values are shown in Figure 3*D* together with a representation of average relative Ca^{2+} permeability of kainate receptors on DRG neurons (as in Fig. 3*A*) from the same-age animals. Individual index of rectification values from P7 DRG neurons are significantly higher than those from P0 and P1 neurons (Fig. 3*C,D*), whereas the relative Ca^{2+} permeability at P7 is significantly lower (Fig. 3*D*).

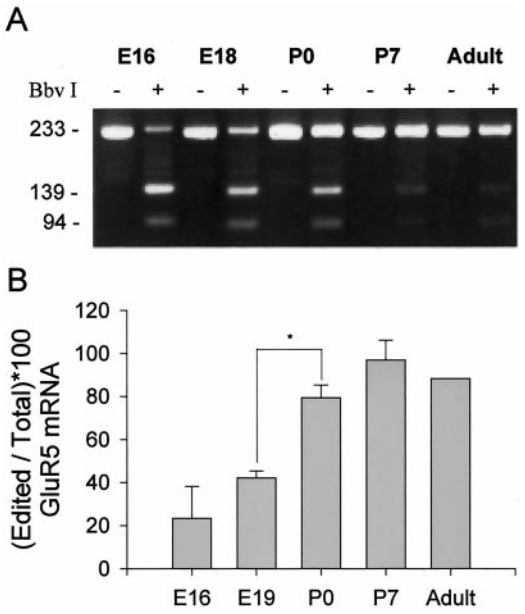

Figure 4. The extent of GluR5 editing sharply increases from embryonic day 16 through the first postnatal week, correlating well with the time course of the decrease in Ca^{2+} permeability. *A*, Unedited GluR5 decreases over the first postnatal week. After reverse transcription-PCR, isolated GluR5 transcripts were digested by the endonuclease *Bbv*I, separated on polyacrylamide–TBE gels, and stained with ethidium bromide for visualization. The presence of two *bottom bands* at 139 and 94 bp represents the unedited GluR5 digested by *Bbv*I. The remaining *band* on the *top* at 233 bp represents the amount of edited GluR5. *B*, The summary of the GluR5-editing assay. The extent of GluR5 editing at the Q/R site is measured by comparing the intensity of the *top bands* of the − *column* (no *Bbv*I added) with that of the + *column* (*Bbv*I added). For each age point, at least three preparations of DRGs were used, except for the adult. The *asterisk* indicates a significant difference (based on Student's *t* test) between E19 and P0 ($p < 0.05$). The error bars indicate the SEM. There is a progressive increase in the extent of editing until it is essentially complete at P7.

GluR5 mRNA-editing assay

A loss of kainate receptor Ca^{2+} permeability in DRG neurons during late embryonic development into the first postnatal week is most simply explained by a change in editing of the GluR5 subunit. To test this hypothesis, we took whole DRGs from rats at different ages, reverse transcribed the mRNA, amplified a portion of the GluR5 subunit incorporating the Q/R site via PCR, and then used the restriction enzyme *Bbv*I to cut this amplified product. Results of this editing assay are shown in Figure 4*A*. The 233 bp band represents the amplified PCR product of GluR5. *Bbv*I was used to assess the extent of GluR5 editing because the unedited fragment can be digested by *Bbv*I whereas the edited fragment cannot (Bernard and Khrestchatisky, 1994). The ratio of the density of the 233 bp band in the presence of *Bbv*I over the density in the absence of *Bbv*I was calculated. This ratio represents the extent of GluR5 editing at the mRNA level. The summary of multiple experiments is shown in Figure 4*B*. The percentage of editing at E16 is 22%, and by the age of P7, most of GluR5 mRNA is edited (95%). In another study, the extent of GluR5 Q/R editing in DRG was reported at only two time points as 11% at E17 and 56% at adult ages (Bernard et al., 1999). Apparent differences in these percentages compared with those we report here could be caused by differences in detection and quantification methods. Our data are consistent with the time course of Ca^{2+} permeability shown physiologically in Figure 3 and support the hypothesis that the developmental increase in the extent of editing at the Q/R site of GluR5 mRNA causes a decrease in the Ca^{2+} permeability and channel conductance of kainate receptors.

Kainate receptors on identified subpopulations of nociceptors

Another explanation for the change of Ca^{2+} permeability of kainate receptors could be the disappearance of a population of neurons that express kainate receptors with high Ca^{2+} permeability and the appearance of a new population of neurons expressing kainate receptors with low Ca^{2+} permeability. To test this possibility, we used several markers to define subpopulations of DRG neurons operationally and then determined which ones express kainate receptors. Labeling was performed using immunocytochemistry for known markers of nociceptors including antibodies to VR1, LA4, LD2, SSEA4, and substance P. VR1 is the protein receptor for noxious heat and is expressed by noxious heat-sensitive nociceptors (Caterina et al., 1997, 2000) projecting to lamina I, II, V, and X in the spinal cord (Tominaga et al., 1998). LA4 labels a subpopulation of DRG neurons that do not express substance P, that display properties of C-fiber-type nociceptors, and that project predominantly to inner lamina II of the superficial dorsal horn (Dodd and Jessell, 1985; Stucky and Lewin, 1999;

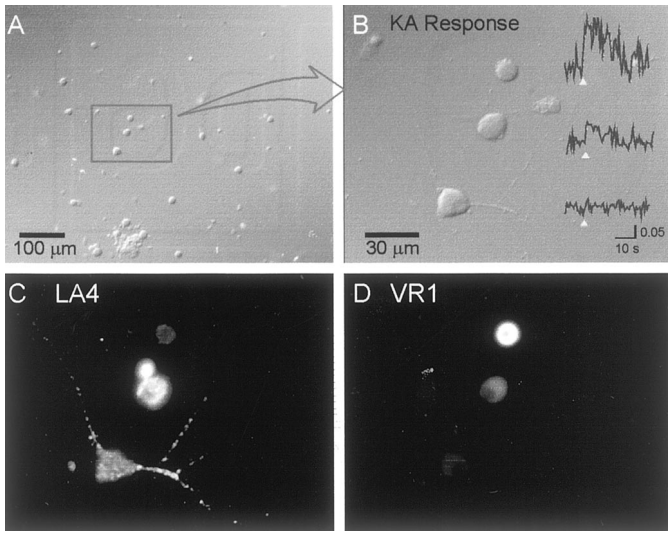

Figure 5. Subpopulations of nociceptors express kainate receptors. Acutely dissociated DRG neurons were plated on glass coverslips with grids. Each grid has unique lettering, which helped us to relocate the same field of view after Ca^{2+} imaging followed by immunostaining with various markers. *A, B*, The area in the *rectangle* in *A* is zoomed in *B*. A field is imaged, and kainate is applied to look for kainate receptor-mediated responses. *Traces* in *B* show that only the *top* two cells had Ca^{2+} responses to 100 μM kainate. *C, D*, The same field of neurons in *B* costained with LA4 and VR1 is shown. The *top* two cells show VR1 expression (*D*), whereas the *bottom* two cells show LA4 expression (*C*).

Fang et al., 2000). These neurons are also identified by the lectin IB4 (see below). LD2 and SSEA4 mark DRG neurons that project predominantly to outer lamina II and laminas I and III, respectively, and are substance P negative (Dodd and Jessell, 1985). Substance P-expressing DRG neurons are nociceptors that project to lamina I and outer lamina II (Todd and Spike, 1993). Marker information is summarized (see Fig. 6*A*).

Figure 5 shows a typical experiment including cells with and without kainate receptors that were labeled with LA4 and VR1. DRG neurons from P1 rat pups were acutely dissociated and plated on glass coverslips pre-etched with labeled grids (Fig. 5*A*; see Materials and Methods). Three neurons shown in the *rectangle* of Figure 5*A* were tested for sensitivity to kainate. The *top* two DRG neurons had kainate-induced changes in $[Ca^{2+}]_i$, whereas the *bottom* cell did not (Fig. 5*B*). After screening multiple fields of cells in this way for the presence of kainate receptors, neurons on each coverslip were fixed and labeled with one or two antibodies. The *top* cell in Figure 5*C* was negative for LA4 but was strongly positive for VR1 (Fig. 5*D*). The *middle* cell showed strong staining for LA4 and intermediate staining for VR1. The *bottom* cell showed no staining for VR1 but was strongly positive for LA4.

In the absence of voltage-clamp control, kainate-induced changes in $[Ca^{2+}]_i$ are mediated by a direct Ca^{2+} influx through Ca^{2+}-permeable kainate receptors, by indirect activation of voltage-gated Ca^{2+} channels, or by both. It follows that detection of DRG neurons expressing Ca^{2+}-impermeable kainate receptors with kainate application requires the presence of voltage-gated Ca^{2+} channels. Therefore, we tested whether all P1 DRG neurons express these channels. We found that 100% of P1 DRG neurons tested for sensitivity to depolarization by 90 mM KCl solution ($n = 120/120$) displayed a robust increase in $[Ca^{2+}]_i$ that was readily blocked by 100 μM $CdCl_2$. Therefore our functional test for the presence of kainate receptors based on $[Ca^{2+}]_i$ elevation was a good indicator of the presence of kainate receptors, regardless of the relative level of Ca^{2+}-permeable receptors.

A

	VR1	LA4	LD2	SSEA4	Sub P
Primary laminar termination site	I, II, V, X	II_i	II_o	I, III	I, II_o
Function/properties	Heat, capsaicin	IB4, FRAP, somatostatin	Some FRAP, somatostatin	Globoseries carbohydrate	peptide

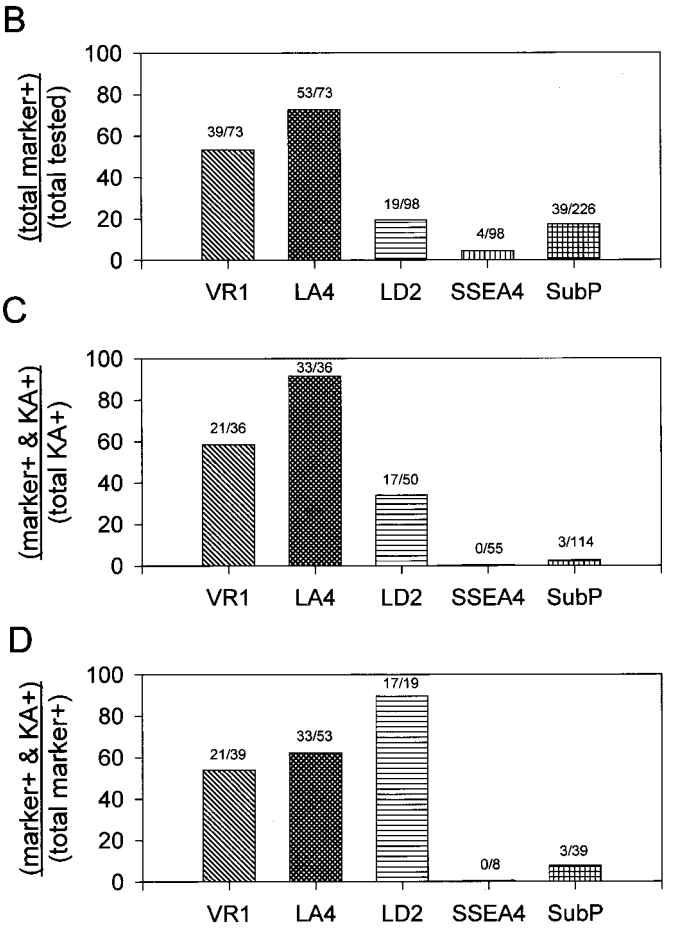

Figure 6. Many LA4- and LD2-positive DRG neurons express kainate receptors, but SSEA4- and substance P-positive DRG neurons do not. *A*, The table summarizes laminar projections and relevant functions or properties of the DRG neurons identified by the individual markers. References are given in Results. *B*, The percentage of total neurons expressing each marker is shown. Only cells tested with kainate application are included. *C*, The percentage of kainate-responsive neurons expressing each marker is shown. Over 90% of the total kainate-responsive neurons also expressed LA4. *D*, The percentage of neurons showing sensitivity to kainate within each labeled subpopulation is shown. Over 90% of the LD2-positive neurons have kainate responses. All data are from P1 acutely dissociated DRG neurons. *Numbers* above each *bar* indicate the number of cells over the total. *FRAP*, Fluoride-resistant acid phosphatase; *SubP*, substance P.

Using this approach, the percentage of neurons within each labeled DRG neuron population expressing kainate receptors and the distribution of differently labeled populations of DRG neurons within the population of kainate-sensitive neurons were determined, and the results are summarized in Figure 6. The distribution of each marker assayed within populations of acutely dissociated DRG neurons tested for sensitivity to kainate is shown in Figure 6*B*. Approximately half of all neurons tested

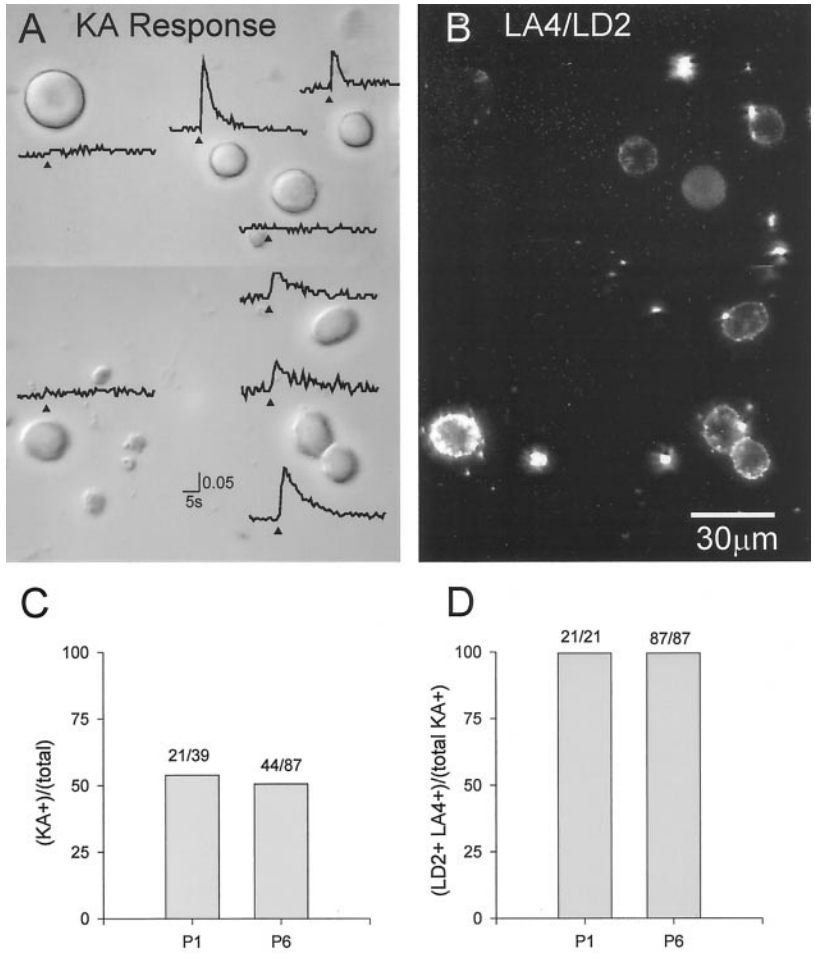

Figure 7. One hundred percent of the DRG neurons expressing kainate receptors are included in the LA4 and LD2-labeled subpopulation both at P1 and P6. *A*, Five of eight P6 DRG neurons show Ca^{2+} responses to kainate, whereas three do not. *B*, The cells were fixed and then stained with both LA4 and LD2. Seven of eight neurons show a positive staining with LA4 and LD2. One large DRG neuron on the *top left* shows negative staining with LA4 and LD2 and no Ca^{2+} response to kainate. *C*, The percentages of kainate-responsive neurons are similar in P1 and P6 DRG neurons. *D*, All of the kainate-responsive neurons show expression of LA4 and LD2 in P1 and P6. The *numbers* above each *bar* represent the number of positive neurons over the total.

showed Ca^{2+} responses to 100 μM kainate (200/397). Of the 73 cells assayed for kainate sensitivity and VR1 expression, approximately half were positive for VR1 (53%). Seventy-two percent of the DRG neurons tested were LA4 positive, the single largest population, whereas LD2, substance P, and SSEA4 each labeled <20% of the neurons tested.

Of the DRG neurons that tested positive for kainate responses, 92% were positive for LA4 (Fig. 6*C*). We have also tested the relationship between LA4 and IB4 labeling of DRG neurons. Ninety-eight percent of IB4-positive neurons (n = 573/584; data not shown) double labeled with LA4, whereas 92% of LA4-positive neurons labeled with IB4. Thus DRG neurons marked by LA4 are virtually identical to those marked by IB4. It follows that the vast majority of the neurons expressing kainate receptors in our acutely dissociated DRG neuron preparations were of the LA4 and IB4 cell types. This would suggest that few if any of the substance P- or SSEA4-positive DRG neurons should have responses to kainate, and this is evident in Figure 6*C*. Essentially none of the cells in these two populations expressed detectable kainate responses. Thirty-four percent of the kainate-responsive neurons were LD2 positive (Fig. 6*C*). Finally, 60% of kainate-responsive neurons were VR1 positive. These data indicate that kainate receptors are expressed by nociceptors, some of which are sensitive to noxious heat.

We then examined what proportion of each labeled subpopulation of DRG neurons expressed kainate receptors (Fig. 6*D*). Sixty-two percent of LA4-positive neurons are kainate responsive. Taken together with the data in Figure 6*C*, this means that although a kainate-responsive neuron is highly likely to be LA4 positive, an LA4-positive neuron is only modestly likely to be kainate responsive. This contrasts with LD2-positive neurons of which 90% are kainate responsive. These data indicate that LD2 is a useful marker for pre-identifying kainate receptor-expressing DRG neurons. We directly tested whether LA4 and LD2 together were able to identify all of the kainate-responsive neurons and found that indeed they marked 100% of the kainate-responsive neurons (see Fig. 7). SSEA4-positive neurons were rare in the acutely prepared DRG neuron populations under study (Fig. 6*B*). Therefore, for the data shown in Figure 6*D*, only four of eight neurons were prelabeled with SSEA4 and then tested for sensitivity to kainate. Figure 6*D* shows that none of the eight SSEA4-positive DRG neurons tested were sensitive to kainate. Finally, only 8% of the substance P-positive cells were sensitive to kainate.

Because LA4 and LD2 identify all of the DRG neurons that express kainate receptors, we used these two markers together to test whether there is a change in the population of DRG neurons expressing kainate receptor over the first postnatal week. Figure 7*A* shows eight DRG neurons from a P6 rat pup, five of which have Ca^{2+} responses to 100 μM kainate. Figure 7*B* shows that seven of eight neurons have positive staining for LA4 and LD2. The one larger cell on the *top left* is negative for LA4 and LD2 and insensitive to kainate. We found that the percentage of kainate-responsive neurons did not change from ~50% between P1 and P6 (Fig. 7*C*). Furthermore, we found that combined staining with LA4 and LD2 marked 100% of the nociceptors

expressing kainate receptor at both P1 and P6 (Fig. 7*D*). These data indicate that changes in the Ca^{2+} permeability of kainate receptors occur in the subpopulation of nociceptors defined by LA4 and LD2.

DISCUSSION

We have directly tested Ca^{2+} permeability of kainate receptors expressed by sensory neurons in DRG. The receptors are mostly Ca^{2+} permeable before birth but switch to being Ca^{2+} impermeable early in the first postnatal week. The time course of this switch correlates with the developmental time course of GluR5 mRNA editing. Using a variety of markers for subpopulations of DRG neurons, we have shown that DRG neurons expressing kainate receptors both at birth and at the end of the first postnatal week are a subpopulation of C-fiber-type nociceptors that projects to lamina II of the spinal cord dorsal horn.

Ca^{2+} permeability of kainate receptors in DRG neurons

Using simultaneous recording of kainate-induced current and Ca^{2+} transients, we have demonstrated for the first time that kainate receptors expressed by embryonic and neonatal DRG neurons are Ca^{2+} permeable. Under voltage clamp, the increase in $[Ca^{2+}]_i$ during kainate application is most likely caused by a direct entry of Ca^{2+} through Ca^{2+}-permeable kainate channels expressed by these neurons. It is unlikely to be caused by Ca^{2+} entry through voltage-gated Ca^{2+} channels because the cells were held at −70 mV and because the increase in $[Ca^{2+}]_i$ was also present in the 0Na/10Ca bath, a condition under which minimal depolarization occurs. It is unlikely to be caused by a Ca^{2+} release from internal stores because the *I–V* relationship was inwardly rectifying in neurons showing kainate-induced Ca^{2+} entry under voltage-clamp conditions. This inward rectification is a hallmark of Ca^{2+}-permeable kainate receptors (Burnashev et al., 1995).

Developmental change in Ca^{2+} permeability of kainate receptors

Our study demonstrates that the rapid decrease in relative Ca^{2+} permeability of kainate receptors over late embryonic and early postnatal times correlates reasonably well with the time course of change in the extent of Q/R editing of GluR5 mRNA. The extent of editing increased from 23% at E16 to 79% at P0 and >97% at P7. By our measure, Ca^{2+} permeability of kainate receptors became uniformly low by P4. Thus there is a close correspondence between the degree of editing and Ca^{2+} permeability. Yet, by analogy to the AMPA receptor in which the inclusion of one edited GluR2 is sufficient to cause low Ca^{2+} permeability of heteromeric AMPA receptors (Geiger et al., 1995; Washburn et al., 1997), the presence of one edited GluR5(R) might be sufficient to make kainate receptors Ca^{2+} impermeable. On the basis of this assumption, it is expected that essentially none of the kainate receptors would be Ca^{2+} permeable at P0 when nearly 80% of the mRNA is edited. Yet all DRG neurons tested at that age showed substantial Ca^{2+} permeability. This suggests that the properties of functional kainate receptors on the plasma membrane of DRG neurons may not directly reflect the mRNA present in the cell because of rate-limiting aspects of receptor protein synthesis and turnover. Alternatively, the subunit configuration of kainate receptors may influence their Ca^{2+} permeability with somewhat different rules than those of AMPA receptors.

GluR5 is the predominant kainate receptor subunit expressed by DRG neurons (Partin et al., 1993). By *in situ* hybridization, the mRNA for GluR5 was shown to be specifically localized in small-diameter DRG neurons (Sato et al., 1993). Smaller amounts of other subunits were also detected including GluR6, GluR7, KA1, and KA2 (Partin et al., 1993). Results from our Q/R-editing experiments suggest that there is a developmentally regulated, dynamic change in the relative ratio of GluR5(Q) and GluR5(R) subunits. In addition, however, other kainate receptor subunits could contribute to a heteromeric receptor configuration with GluR5, modifying channel properties (Herb et al., 1992; Cui and Mayer, 1999).

Identity of DRG neurons expressing functional kainate receptors

Huettner (1990) was the first to report the presence of kainate receptors on a subpopulation of DRG neurons that he described as having intermediate and small diameters. Taken together with the observation that the agonist kainate depresses compound action potentials recorded from C-fibers (Agrawal and Evans, 1986), these data suggest that kainate receptors are expressed by nociceptors. Using a panel of markers for different subpopulations of nociceptors, we have tested which subpopulations of DRG neurons express functional kainate receptors and have found them to be expressed on substance P-negative, SSEA4-negative, LA4 and IB4-positive, and LD2-positive DRG neurons. LA4 and IB4-positive neurons are C-fiber-type nociceptors as shown by Fang et al. (2000) who directly demonstrated that all IB4-labeled DRG neurons are C-fiber neurons.

Although LA4 alone accounts for 92% of the DRG neurons expressing kainate receptors, LA4 and LD2 together account for 100% of these neurons, indicating that LD2 must label a population of DRG neurons not labeled by LA4. The LA4-positive neurons represent a larger part of the total DRG population than do LD2-positive neurons in the ganglion from adult rats (50 vs 25%) (Dodd and Jessell, 1985) and in our acutely dissociated preparation from newborn pups (72 vs 19%; see Fig. 6). The LA4-positive neurons include nearly all of the FRAP-positive and all of the somatostatin (SOM)-positive afferents. The LD2-positive neurons also include all of the SOM-positive afferents and a few of the FRAP-positive afferents (Dodd and Jessell, 1985). Thus it is likely that essentially all DRG neurons expressing functional kainate receptor are FRAP and SOM positive.

C-fiber neurons expressing kainate receptors and projecting to lamina II are not expected to be sensitive to a uniform sensory modality. In a double-label experiment, 58% of LA4-positive neurons were positive for VR1 (our unpublished observation). This distribution is comparable with the findings of Guo et al. (1999) who reported 67% of IB4-positive neurons showing an overlap with VR1 in intact ganglia. In our experiments, 60% of kainate-responsive cells were positive for antibody to VR1, and 71% of the kainate-sensitive neurons were sensitive to capsaicin, an agonist for VR1. Only these capsaicin-sensitive, VR1-positive neurons are likely to be nociceptors sensitive to noxious heat (Caterina et al., 2000), indicating that there is more than one sensory modality represented in this LA4-positive, kainate receptor-positive population of neurons.

Possible role of Ca^{2+}-permeable kainate receptors in developing sensory axons

In a recent study, the physiological significance of Q/R editing of GluR5 was tested by generating a mouse with genetically altered GluR5 so that all GluR5 mRNA was in the GluR5(R) form (Sailer et al., 1999). No developmental abnormalities were de-

tected in these animals, nor did they show deficits in the behavioral tests conducted in the study, although there was a sixfold decrease in kainate current density in postnatal DRG neurons. However, the nociceptive testing in these experiments was limited, leaving open the possibility of a role for Ca^{2+}-permeable kainate receptors in nociceptor development.

The timing of the switch of Ca^{2+} permeability and of the sharp increase in the extent of GluR5 Q/R editing coincides with important changes in the invasion of C-fiber primary afferent axons in the spinal cord dorsal horn and their synaptic termination there. Although large-diameter dorsal root afferent collaterals begin to grow into the dorsal horn at E15 in the rat (Smith, 1983; Fitzgerald et al., 1991), C-fibers, including those expressing IB4, grow into the superficial dorsal horn from approximately E17 and later (Fitzgerald, 1987; Mirnics and Koerber, 1995; Jackman and Fitzgerald, 2000). Substance P- and FRAP-containing fibers are clearly evident in laminas I and II by P1 (Fitzgerald and Gibson, 1984). Indeed, C-type afferent terminals are observed to make simple synaptic connections in lamina IIi by P0, and by P2 many express FRAP. Some of these mature into glomerular endings by approximately P5 (Pignatelli et al., 1989). Although the fibers are present in the dorsal horn, robust C-fiber-evoked responses are not observed during the first postnatal week, whereas A-fiber-evoked responses are readily observed in neonates (Jennings and Fitzgerald, 1998). Because the kainate receptors are expressed by a subpopulation of sensory neurons projecting to lamina II of the dorsal horn and Ca^{2+} permeability of the receptors switches off at approximately the time of birth and over the first postnatal week, this change might be related to the C-fiber invasion of spinal cord and the formation of early synaptic contacts between C-fibers and target dorsal horn neurons.

In adult rat spinal cord, Aβ afferents are restricted to laminas III and IV, whereas in the neonate, the Aβ terminals extend dorsally into laminas I and II (Fitzgerald et al., 1994; Mirnics and Koerber, 1995). The Aβ fibers then gradually withdraw from the superficial laminas over the first 3 postnatal weeks. C-fibers, on the other hand, grow specifically to laminas I and II before birth and for a considerable postnatal period; these laminas are occupied by both A- and C-fiber terminals (Fitzgerald et al., 1994). If Ca^{2+}-permeable kainate receptors are expressed on the growth cones of growing C-fiber axons, they might function as a signal for C-fibers to stop in lamina II and form synapses. One possible source of glutamate for triggering this stop signal could be from Aβ fiber terminals that are present in lamina II before birth. Later, kainate receptors would then switch to a Ca^{2+}-impermeable form to serve yet another function, possibly as a modulator of synaptic release of glutamate from central terminals of primary afferents in the dorsal horn (Lee et al., 1999; Kerchner et al., 2001), as reported for mossy fibers in the hippocampus (Kamiya and Ozawa, 1999; Schmitz et al., 2000).

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