Kainate Receptor Subunits Underlying Presynaptic Regulation of Transmitter Release in the Dorsal Horn

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Presynaptic kainate (KA) receptors regulate synaptic transmission at both excitatory and inhibitory synapses in the spinal cord dorsal horn. Previous work has demonstrated pharmacological differences between the KA receptors expressed by rat dorsal horn neurons and those expressed by the primary afferent sensory neurons that innervate the dorsal horn. Here, neurons isolated from mice deficient in the KA receptor subunit were used to evaluate the contributions of glutamate receptor subunit 5 (GluR5) and GluR6 to the presynaptic control of transmitter release and to KA receptor-mediated whole-cell currents in these two cell populations. Deletion of *GluR6* produced a significant reduction in KA receptor-mediated current density in dorsal horn neurons, whereas *GluR5* deletion caused no change in current density but removed sensitivity to GluR5-selective antagonists. Presynaptic modulation of inhibitory transmission between dorsal horn neurons was preserved in cells from either GluR5- or GluR6-deficient mice. In DRG neurons, in contrast, *GluR5* deletion abolished KA receptor function, whereas deletion of *GluR6* had little effect on peak current density but increased the rate and extent of desensitization. These results highlight fundamental differences in KA receptor physiology between the two cell types and suggest possible strategies for the pharmacological modulation of nociception.

Key words: kainate; glutamate receptor; presynaptic; GluR5; GluR6; spinal cord; dorsal horn; dorsal root ganglion; sensory transmission

Kainate (KA) receptors are multisubunit ion channels that play an important role as postsynaptic mediators of transmission at a variety of excitatory synapses in the CNS. In addition, recent work indicates that presynaptic KA receptors may serve to regulate transmitter release from both excitatory and inhibitory nerve terminals (for review, see Chittajallu et al., 1999; Frerking and Nicoll, 2000; Lerma et al., 2001). There are five different subunits that can contribute to KA receptors (Hollmann and Heinemann, 1994), including glutamate receptor subunit 5 (GluR5), GluR6, and GluR7, which can form functional homomeric receptors, and KA1 and KA2, which combine in heteromeric receptors but do not form functional ion channels on their own. Genetic deletions of GluR5 (Mulle et al., 2000) and GluR6 (Mulle et al., 1998) have revealed important and distinct roles for these subunits in synaptic transmission and plasticity in the hippocampus (Bureau et al., 1999; Contractor et al., 2000, 2001) and striatum (Chergui et al., 2000). Much less is known about the roles of individual KA receptor subunits in other parts of the nervous system.

In the spinal cord dorsal horn, presynaptic KA receptors regulate transmission at both excitatory and inhibitory synapses (Huettner et al., 2002). At excitatory primary afferent sensory synapses, KA receptors expressed by a subset of DRG neurons are located on presynaptic terminals (Hwang et al., 2001), where they regulate glutamate release (Kerchner et al., 2001b). At inhibitory synapses within the dorsal horn, presynaptic KA receptors, which respond to glutamate released from dorsal root sensory fibers, regulate GABA and glycine release by direct depolarization of interneuron terminals (Kerchner et al., 2001a). In addition to these presynaptic receptors on excitatory and inhibitory terminals, KA receptors also are found on the postsynaptic membrane of neurons that respond to high-threshold dorsal root fiber stimulation (Li et al., 1999).

It is not yet known which KA receptor subunits underlie these distinct synaptic functions in the dorsal horn. In previous work, a pharmacological difference was identified between KA receptors on rat DRG neurons, which were activated and potently desensitized by the GluR5-preferring agonist (*RS*)-2-amino-3-(3-hydroxy-5-tertbutylisoxazol-4-yl)propanoic acid (ATPA), and those on rat dorsal horn neurons, which were largely insensitive to ATPA (Kerchner et al., 2001b; Wilding and Huettner, 2001). These results are consistent with the prevalence of GluR5 mRNA in DRG but not dorsal horn neurons (Partin et al., 1993; Sato et al., 1993; Tölle et al., 1993). However, the pharmacology of ATPA is not definitive in this regard, because it also activates some heteromeric KA receptors that do not contain the GluR5 subunit (Paternain et al., 2000). In addition, it remains unclear which subunits underlie KA responses in dorsal horn neurons. In this study, we make use of mice deficient in the GluR5 and GluR6 KA receptor subunits, as well as antagonists selective for the GluR5 subunit, to test whether GluR5 and GluR6 are required for the assembly of functional KA receptors in the dorsal horn.

MATERIALS AND METHODS

Mice. The protocols for handling animals were approved by the Animal Studies Committee at Washington University. *GluR5*−/− and *GluR6*−/− mice were obtained as gifts from Stephen F. Heinemann (Salk Institute,

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San Diego, CA), and wild-type mice were C57BL/6 × 129S6/SvEv hybrids purchased from Taconic (Germantown, NY).

Primary neuronal culture. Dorsal horn neurons were taken from postnatal mice killed by decapitation. The spinal cord was removed to a dish containing Earle's buffer, and the dorsal third of the cord was dissected and incubated for 30–90 min at 30–35°C in oxygenated Earle's buffer containing papain (Huettner and Baughman, 1986; Wilding and Huettner, 1997). Cells were dissociated mechanically in bovine serum albumin and ovomucoid, both at 1 mg/ml, and plated onto 35 mm culture dishes coated with matrigel (Becton Dickinson, Bedford, MA). For DRG/spinal cocultures, the DRGs were isolated, treated as described above, and plated with dorsal horn neurons onto large islands (~200 μm square), created by drawing a grid of agarose (1.5 mg/ml) on the bottom of 35 mm dishes, which were then sprayed with collagen or a mixture of poly-DL-ornithine (0.2 mg/ml) and laminin (6 μg/ml). In some experiments, DRG soma were isolated and used for experiments within 24 hr (Wilding and Huettner, 1995). Long-term cultures were maintained at 37°C in a humidified, 5% CO_2 incubator in Eagle's minimal essential medium (supplemented with 20 mM glucose, 0.5 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 4% rat serum) (nerve growth factor was added when DRGs were plated), treated after 4 d *in vitro* with 10 μM cytosine β-D-arabinofuranoside, and used for experiments *in vitro* between 7 and 35 d.

Electrophysiology. On the stage of an Axiovert 25 inverted microscope (Zeiss, Thornwood, NY), cultures were bath-perfused with Tyrode's solution, containing (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, and 10 HEPES, pH 7.4, with NaOH. In experiments testing NMDA receptor-mediated responses, a Tyrode's solution lacking $MgCl_2$ was used. Rapid agonist applications to characterize KA receptors were made from a multibarreled pipette (Wilding and Huettner, 1997) fed by solution reservoirs maintained under 8–10 psi of static air pressure. During recordings of synaptic currents, neurons were under constant local gravity-fed perfusion from a quartz glass pipette (inner diameter, 300 μm) connected to a manifold with <1 μl dead space (ALA Scientific Instruments, Inc., Westbury, NY). The local perfusion solutions consisted of Tyrode's solution plus various pharmacological agents. When measuring KA-evoked currents, 300 μM KA was used, except in experiments testing blockade by (3*S*,4a*R*,6*S*,8a*R*)-6-(4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid (LY382884) or (3*S*,4a*R*,6*R*,8a*R*)-6-{2-[1(2)H-tetrazol-5-yl]ethyl}-decahydroisoquinoline-3-carboxylic acid (LY293558), in which case 50 μM KA was used.

Whole-cell recordings were established using heat-polished pipettes pulled from filamented borosilicate capillary tubes (Warner Instruments, Hamden, CT) with a tip resistance of 3–8 MΩ when filled with a solution containing (in mM): 140 $CsCH_3SO_3$, 5 CsCl, 5 $MgCl_2$, 10 EGTA, 10 HEPES, 5 Mg-ATP, and 1 Li-GTP, pH 7.4, with CsOH. Neurons were typically held at −70 mV (for measuring excitatory currents) or 0 mV (for inhibitory currents). Neither series resistance compensation nor cell membrane capacitance neutralization were routinely applied, but both were monitored throughout experiments. Recorded currents were filtered at 2 kHz, digitized at 10 kHz, and stored in a personal computer for display and analysis with an Axopatch 200B amplifier, Digidata 1320 interface, and the pClamp 8 software suite (Axon Instruments, Foster City, CA).

Extracellular stimulation of synaptic currents was achieved with the S48 single-channel stimulator and SIU5 stimulus isolation unit (Grass Instruments, Inc., West Warwick, RI) connected to a bipolar stimulating electrode, constructed with two Ag/AgCl wires immersed in Tyrode's solution within a τ glass electrode, which was pulled and heat-polished to a final tip diameter of ~10–20 μm. This stimulus electrode was placed against the cell body of a neuron close to the recorded cell. Experiments were included only when evoked postsynaptic currents occurred at a fixed latency after stimulation. Typically, synaptic stimulation was delivered every 15 sec, in the case of NMDA receptor-mediated EPSCs, or every 5 sec, in the case of IPSCs.

Pharmacology. All experiments were conducted in the presence of the AMPA receptor-selective antagonist (±)-4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine (SYM2206) (100 μM) (Li et al., 1999; Wilding and Huettner, 2001) to permit selective KA receptor activation. In studies of inhibitory synaptic transmission, DL-2-amino-5-phosphono-pentanoic acid (25 μM) was also present. In studies of excitatory synaptic transmission, bicuculline (10 μM) and strychnine (1 μM) were also present. All compounds were purchased from Sigma (St. Louis, MO), except ATPA and SYM2206 (Tocris Cookson, Ellisville, MO) and LY382884 and LY293558, which were obtained as gifts from Eli Lilly and Co. (Greenfield, IN).

Data analysis. Data are presented as means ± SEM. To detect significant differences between two means, a paired *t* test or signed rank test was used. For comparison of multiple groups, a one-way ANOVA was performed with the Student–Newman–Keuls test for *post hoc* comparison. Cumulative probability plots were compared with the Kolmogorov–Smirnov test. In all cases, $p < 0.05$ was considered significant. Time constants for KA receptor desensitization were determined by fitting a sum of two exponential functions plus a constant to the falling phase of evoked current.

RESULTS

KA receptors expressed by dorsal horn neurons

Previous physiological recordings have documented the expression of functional KA receptors by nearly all rat dorsal horn neurons in culture (Kerchner et al., 2001b; Wilding and Huettner, 2001) and by a significant proportion of neurons in acute spinal cord slices (Li et al., 1999; Kerchner et al., 2001a). However, anatomical studies of KA receptor subunit distribution have not conclusively established the composition of native KA receptors in this region (Tölle et al., 1993; Petralia et al., 1994) (see Discussion). In previous work on rat dorsal horn neurons (Kerchner et al., 2001b; Wilding and Huettner, 2001), we showed that whereas native KA receptors were activated reliably by KA application, the GluR5-selective compound ATPA triggered only small currents in a minority of cells and failed to cross desensitize spinal receptors to activation by KA (Kerchner et al., 2001b; Wilding and Huettner, 2001).

As shown in Figure 1, cultured dorsal horn neurons from wild-type mice also express functional KA receptors. To evaluate the contribution of GluR5 in wild-type cells, we tested the sensitivity of KA-evoked currents to cross desensitization by ATPA or inhibition by the GluR5-selective compounds LY382884 and LY293558 (Bleakman et al., 1996). Similar to results in rat dorsal horn cells, ATPA (30–100 μM) evoked much smaller peak currents than KA (50–300 μM) (Fig. 1*D*) and caused little to no cross desensitization of receptors in spinal neurons from wild-type mice (Fig. 1*E*). However, 10 μM LY382884 (Fig. 1*A,D*) and LY293558 (data not shown) produced significant inhibition of KA-evoked currents. The slow rise in current after agonist onset likely reflects competitive displacement by KA of the antagonist, which was present continuously. Because any receptors that were not affected by the antagonist would be expected to contribute an instantaneous rise in current at agonist onset, the appearance of little instantaneous inward current in most recordings (Fig. 1*A*) suggests that the majority of surface KA receptors were sensitive to the drug. In experiments on wild-type neurons, the instantaneous current ranged from 0.5 to 53% of control peak current, with a mean of 18.6 ± 5.3% ($n = 12$).

Mice deficient in either GluR5 or GluR6 were used to evaluate further the specific contributions of these subunits to KA receptors in dorsal horn neurons. Cultured neurons isolated from *GluR5−/−* mice exhibited robust responses to KA (Fig. 1*A*), with current densities similar to those recorded from wild-type neurons (Fig. 1*B,C*). ATPA evoked little or no current (Fig. 1*D*) and had no effect on responses to KA (Fig. 1*E*). More importantly, KA receptor-mediated currents in *GluR5−/−* cells were completely insensitive to GluR5-selective antagonists (Fig. 1*A,D*). In contrast, dorsal horn neurons from *GluR6−/−* mice exhibited

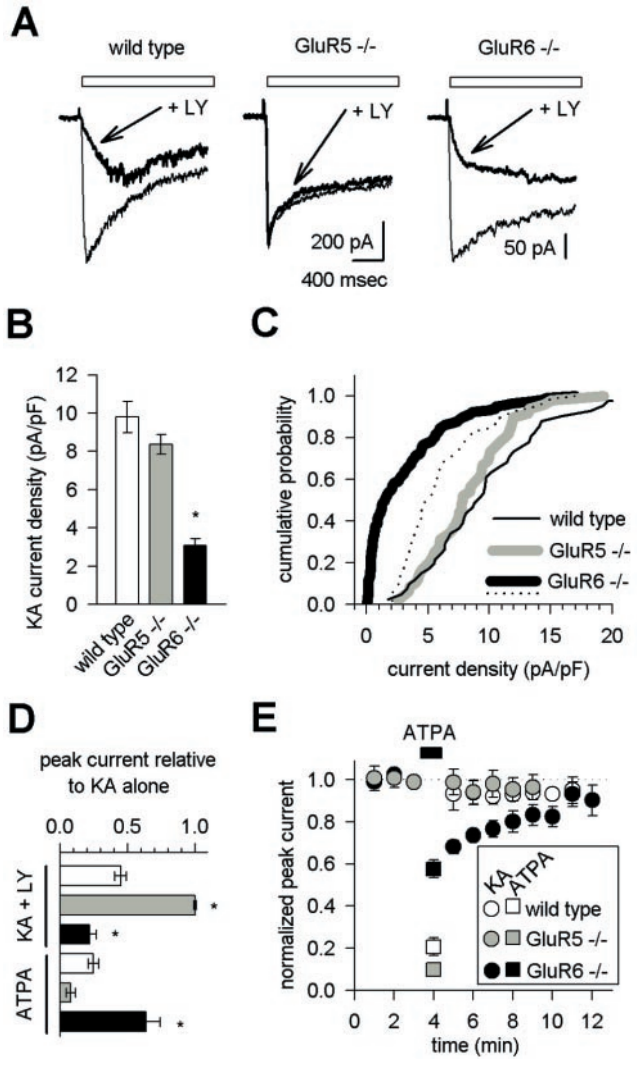

Figure 1. Whole-cell currents in dorsal horn neurons from wild-type and knock-out mice. *A*, Superimposed currents evoked by KA in the absence (*thin line*) and presence (*thick line*) of 10 μM LY382884 (*LY*). *Open bars* indicate the period of agonist exposure. *B*, KA-evoked current density (measured by dividing peak current amplitude by whole-cell capacitance for each cell) was similar in dorsal horn neurons from wild-type mice ($n = 41$ cells) and *GluR5*−/− mice ($n = 48$) but was significantly smaller in cells from *GluR6*−/− mice ($n = 115$). Although KA triggered inward currents (with negative amplitudes), current densities are shown as absolute values in this figure and in Figure 3. *Significantly different from wild type. *C*, Cumulative probability as a function of current density illustrates the prevalence of *GluR6*−/− cells with little or no KA receptor-mediated current. Curves for wild-type and *GluR5*−/− cells were not significantly different ($p = 0.524$), whereas the curve for *GluR6*−/− cells was different from both ($p < 0.0005$; Kolmogorov–Smirnov test). The *dotted curve* shows cumulative probability data for the subset of *GluR6*−/− cells with current density >2.0 pA/pF. This curve was also significantly different from both the *GluR5*−/− and wild-type curves ($p < 0.0005$). *D*, The relative amplitude of peak currents elicited by KA in the continuous presence of 10 μM LY382884 [10 μM LY293558 was used in some experiments (Bleakman et al., 1996)] or by 30 μM ATPA alone are compared with those elicited by KA alone in wild-type, *GluR5*−/−, or *GluR6*−/− neurons ($n = 6$–20 cells per observation). GluR5-selective antagonists blocked KA currents, and ATPA alone triggered currents in neurons from wild-type and *GluR6*−/− but not *GluR5*−/− mice. *Significantly different from wild type. *E*, Cross desensitization of peak current evoked by 300 μM KA resulting from a 2 sec exposure to 100 μM ATPA. Agonists were applied once per minute. *Squares* plot the peak current evoked by ATPA; *circles* plot the current evoked by KA in wild-type ($n = 9$), *GluR5*−/− ($n = 5$), and *GluR6*−/− ($n = 11$) cells. *Dotted line* indicates 100% of normalized peak current.

variable sensitivity to KA. In approximately one-half of the *GluR6*−/− neurons recorded, exposure to KA produced no significant change in the holding current (<1–2 pA/pF); in most of the remaining cells, KA-evoked currents were significantly smaller than for wild-type and *GluR5*−/− neurons (Fig. 1*B,C*). LY382884 blocked a greater proportion of current in *GluR6*−/− than in wild-type cells (Fig. 1*A,D*); instantaneous current in the presence of LY382884 was 2.2 ± 0.9% ($n = 8$) of control peak current for *GluR6*−/− neurons. In addition, a greater proportion of KA receptor-mediated current could be evoked by ATPA in *GluR6*−/− cells than in wild-type cells (Fig. 1*D,E*). The absolute density of ATPA-evoked current was slightly, albeit not significantly, greater in our recordings from 20 *GluR6*−/− cells than in 21 wild-type neurons (3.7 ± 0.9 pA/pF for *GluR6*−/−; 2.9 ± 0.6 pA/pF for wild type). Moreover, exposure of *GluR6*−/− cells to ATPA produced a partial cross desensitization of currents evoked by KA, with recovery occurring over the course of several minutes (Fig. 1*E*). Collectively, these results suggest that KA receptors in cultured murine dorsal horn neurons incorporate both the GluR5 and GluR6 subunits. Although *GluR5* deletion had no effect on current density relative to wild type, *GluR6* deletion reduced or eliminated functional KA receptor expression in most cells, suggesting that GluR6 is more important than GluR5 for the assembly of functional KA receptors in dorsal horn neurons.

KA receptor subunits underlying presynaptic regulation of GABA/glycine release

Presynaptic KA receptors on rat dorsal horn interneuron terminals trigger action potential-independent GABA and glycine release (Kerchner et al., 2001a). We observed a similar effect in cells from wild-type mice, in which KA (10 μM) elevated the frequency of tetrodotoxin (TTX)-insensitive miniature IPSCs (mIPSCs) to 370 ± 60% of control ($n = 8$; $p < 0.001$). If subunit composition is the same for presynaptic KA receptors as for receptors on the cell body, then our observation that *GluR5* deletion had little effect on whole-cell KA-evoked current density (Fig. 1*B,C*) suggests that KA application should affect inhibitory transmission similarly in wild-type and *GluR5*−/− cells. Indeed, exposure to KA (10 μM) triggered a comparable increase in mIPSC frequency (Fig. 2*A,B*) in cultured *GluR5*−/− dorsal horn neurons and in wild type. ATPA (2 μM) was less effective than KA at eliciting GABA/glycine release in wild-type cultures, and it showed no activity in *GluR5*−/− cells. (Fig. 2*B*).

In contrast, *GluR6* deletion, which resulted in lower KA-evoked current densities (see above), might be expected to hinder KA-induced GABA/glycine release (Fig. 1*B,C*). In some recordings from *GluR6*−/− neurons, KA produced no change in mIPSC frequency. On average, the effect of KA was reduced by nearly 70% in *GluR6*−/− cells relative to wild type. Consistent with the observation that ATPA evoked a larger proportion of KA receptor-mediated current in *GluR6*−/− neurons than in wild-type cells (Fig. 1*D*), ATPA also stimulated a greater increase in GABA/glycine release in *GluR6*−/− cultures than in wild type (Fig. 2*A,B*). The larger effect of ATPA versus KA in *GluR6*−/− cultures may reflect activation of a greater proportion of receptors by 2 μM ATPA than by 10 μM KA, consistent with the 20-fold lower EC_{50} of ATPA compared with KA at native receptors on DRG neurons (0.6 vs 12 μM, respectively) (Clarke et al., 1997).

Presynaptic KA receptors also mediate a reduction in action potential-evoked inhibitory transmission between rat dorsal horn neurons, in a mechanism involving $GABA_B$ receptor activation (Kerchner et al., 2001a). KA (3 μM) depressed the amplitude of

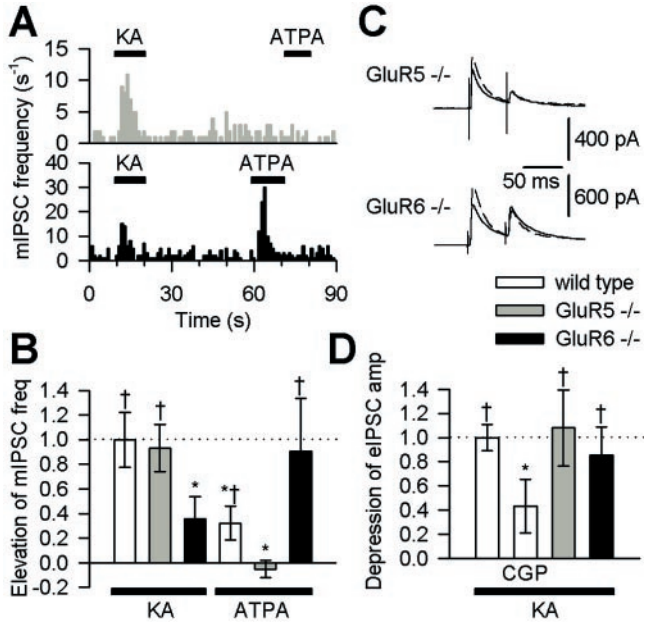

Figure 2. Neither GluR5 nor GluR6 wholly accounts for presynaptic KA receptors on dorsal horn interneurons. *A, B*, The effects of 10 μM KA and 2 μM ATPA on mIPSC frequency (recorded in the presence of 0.5 μM TTX) are compared in cultures of wild-type (n = 8 KA recordings, 8 ATPA), *GluR5*−/− (n = 28 KA, 11 ATPA), and *GluR6*−/− (n = 20 KA, 5 ATPA) dorsal horn neurons. *A*, Representative experiments for *GluR5*−/− (*top*) and *GluR6*−/− (*bottom*) cells. *B*, Frequency (quantified during the first 4 sec of agonist exposure) is normalized to the value in wild-type cultures in the presence of KA (*dotted line*). *Significantly different from the action of KA in wild-type cultures. †Significantly different from baseline frequency in the absence of KA or ATPA. *C, D*, A 3 μM concentration of KA reduced eIPSC amplitude similarly in wild-type (n = 4), *GluR5*−/− (n = 4), and *GluR6*−/− (n = 14) dorsal horn neurons. KA action was reduced by the $GABA_B$ receptor antagonist CGP55845 (*CGP*; 10 μM). Traces from representative recordings (*C*) also document an increase in the paired-pulse ratio between baseline (*dashed line*) and KA (*solid line*) conditions. *D*, Values normalized to the degree of KA-induced suppression of eIPSC amplitude in wild-type cultures (*dotted line*). *Significantly different from KA action in wild-type cultures. †Significantly different from baseline responses in the absence of KA.

IPSCs evoked by extracellular stimulation [evoked IPSCs (eIPSCs)] between mouse dorsal horn neurons to 66 ± 4% of control (n = 4; p = 0.029), an effect that was significantly reduced by the $GABA_B$ receptor antagonist (2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845) (Fig. 2*D*). As expected, this action of KA was not affected by *GluR5* deletion (Fig. 2*C,D*); however, surprisingly, this action was also preserved in *GluR6*−/− cultures (Fig. 2*C,D*). The ability of the *GluR6* deletion to reduce KA action on mIPSCs but not eIPSCs may suggest that a subtle increase in ongoing GABA/glycine release was sufficient to cause $GABA_B$ autoreceptor activation (Kerchner et al., 2001a). Thus, neither GluR5 nor GluR6 wholly accounts for presynaptic KA receptors on mouse dorsal horn inhibitory neurons.

KA receptors expressed by DRG neurons

Although there is evidence for the expression of all five KA receptor subunits in DRGs (Partin et al., 1993; Petralia et al., 1994), both Northern blot analysis (Partin et al., 1993) and pharmacology data (Swanson et al., 1998; Kerchner et al., 2001b; Wilding and Huettner, 2001) point to the predominance of

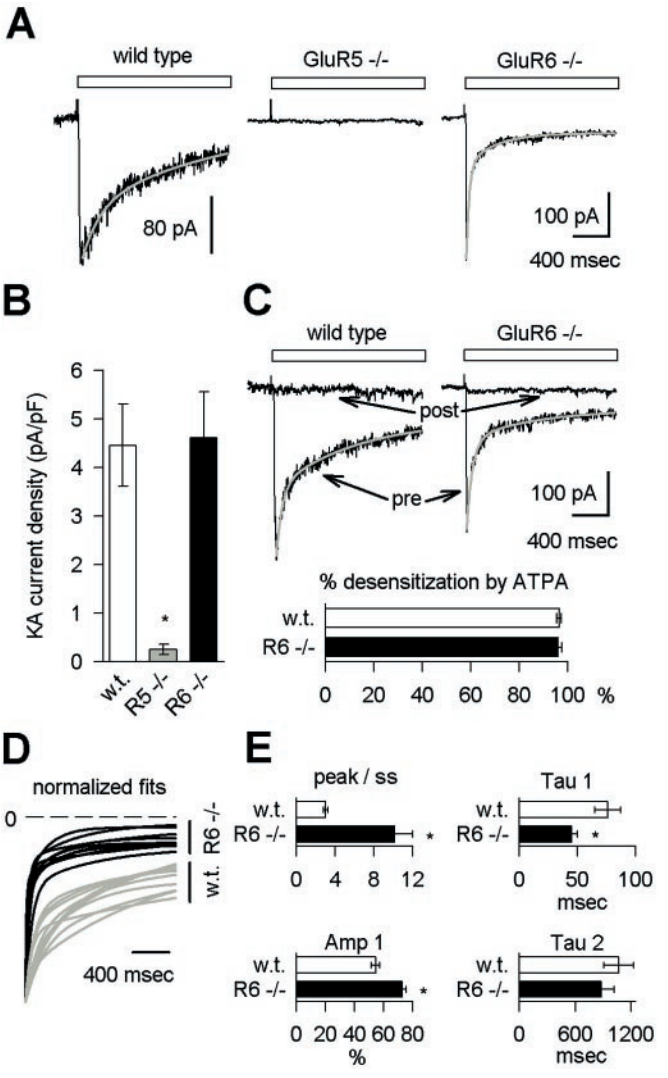

Figure 3. GluR5 is required for functional KA receptor expression in DRG neurons. *A*, Whole-cell currents evoked by 300 μM KA in freshly dissociated DRG neurons from wild-type, *GluR5*−/−, and *GluR6*−/− mice. *Open bars* indicate the periods of agonist exposure. *Smooth curves* are the best fits of the sum of two exponentials plus a constant. *B*, Peak current density was significantly reduced in cells from *GluR5*−/− mice (n = 28) relative to wild-type (*w.t.*) (n = 62) and *GluR6*−/− (n = 60) mice. *Significantly different from wild type. *C*, Superimposed traces show currents evoked by 300 μM KA before (*pre*) and after (*post*) exposure to 500 nM ATPA for 2 sec. The percentage of steady-state cross desensitization by ATPA is plotted for five wild-type and six *GluR6*−/− cells. *D*, Best fits of the sum of two exponentials plus a constant for 10 wild-type and 10 *GluR6*−/− cells are shown normalized to the same initial peak. *Dashed line* indicates zero current level. *E*, Plots compare the ratio of peak to steady-state current for wild-type (n = 27) and *GluR6*−/− (n = 36) cells, as well as parameters from the best fits to the time course of desensitization (n = 16 wild type and 26 *GluR6*−/−), including the amplitude of the first exponential (*Amp 1*) and the time constants of the first (*Tau 1*) and second (*Tau 2*) exponential functions. *Significantly different from wild type.

GluR5. In addition, the report describing the initial generation of *GluR5*−/− mice (Mulle et al., 2000) found a lack of KA receptor-mediated currents in 17 freshly dissociated DRG neurons from these mice, suggesting that the expression of GluR5 was essential for the production of functional receptors by DRG cells. As shown in Figure 3, our results confirm the observations of Mulle et al. (2000): in only 2 of 28 GluR5−/− DRG neurons did rapid

exposure to KA produce a detectable current, and the currents in both of those cells were small (<55 pA). In contrast, KA elicited currents in the majority of wild-type (47 of 62) and *GluR6−/−* (39 of 60) neurons tested. As observed previously in rat DRG cells (Kerchner et al., 2001b; Wilding and Huettner, 2001), brief exposure to ATPA caused profound cross desensitization of currents evoked by KA in both wild-type and *GluR6−/−* neurons (Fig. 3*C*). However, the currents evoked by KA in *GluR6−/−* cells unexpectedly showed more rapid and more complete desensitization than in cells from wild-type mice (Fig. 3*A,C,D*). The ratio of peak to steady-state current (peak/ss) was significantly greater for *GluR6−/−* cells than for wild-type cells (Fig. 3*E*, *peak/ss*). In addition, the initial time constant for current desensitization (see Materials and Methods) was shorter in *GluR6−/−* cells (Fig. 3*E*, *Tau 1*), and the relative contribution by the faster exponential was greater for *GluR6−/−* cells than for wild type (Fig. 3*E*, *Amp 1*). Thus, our results support a requirement for GluR5 in the assembly of functional KA receptors in DRG cells and identify a potential role for GluR6 in modulating receptor kinetics.

KA receptor subunits underlying presynaptic regulation of DRG to spinal transmission

Activation of presynaptic KA receptors on DRG cells reduces glutamatergic transmission onto dorsal horn target neurons in rats (Kerchner et al., 2001b). KA application had a similar effect in DRG/spinal neuron cocultures from wild-type mice (Fig. 4). KA (10 μM) reduced the amplitude of NMDA receptor-mediated EPSCs evoked in dorsal horn neurons by extracellular stimulation directed at DRG cell bodies to 52 ± 2% of the control value ($n = 4$; $p = 0.029$). Based on the results for agonist-evoked currents in subunit-deficient mice (Fig. 3), we anticipated that *GluR5* but not *GluR6* deletion would disrupt the presynaptic regulation of DRG to spinal transmission by KA. To our surprise, KA action was reduced to a similar extent, but not eliminated, by the deletion of either subunit. To explain the action of KA in *GluR5−/−* cocultures, we initially speculated that GluR6-containing somatodendritic receptors on dorsal horn neurons might shunt postsynaptic current, thereby reducing EPSC amplitude (Frerking et al., 1999); however, at 10 μM, KA caused no significant change in input resistance in *GluR5−/−* dorsal horn neurons (88 ± 11% of control; $n = 5$; $p = 0.31$) (Kerchner et al., 2001b).

An alternative hypothesis explaining why *GluR5* and *GluR6* deletions each partially reduced KA action is that the extracellular stimulating electrode may have activated not only a presynaptic DRG neuron but also nearby excitatory dorsal horn neuronal cell bodies or axons. In this scenario, KA, which suppresses both DRG-to-spinal and spinal-to-spinal excitatory transmission (Kerchner et al., 2001b), may inhibit composite EPSCs by activating presynaptic GluR5-containing KA receptors on DRG neurons as well as presynaptic GluR6-containing KA receptors on dorsal horn neurons. This hypothesis would account for the observed reduction in KA action in both *GluR5−/−* and *GluR6−/−* cocultures relative to wild type. Supporting this hypothesis, in mixed cocultures of *GluR5−/−* DRGs and *GluR6−/−* dorsal horn neurons, KA had no effect on EPSCs (Fig. 4). Further establishing that GluR5 is required for KA modulation of DRG-to-spinal transmission, ATPA strongly suppressed DRG to spinal transmission in wild-type and *GluR6−/−* cultures, but had no significant effect in *GluR5−/−* cocultures and in mixed *GluR5−/−* DRG/*GluR6−/−* spinal cocultures (Fig. 4*B*). Finally, LY382884 blocked the action of KA in cocultures from *GluR6−/−* mice (Fig. 4*B*).

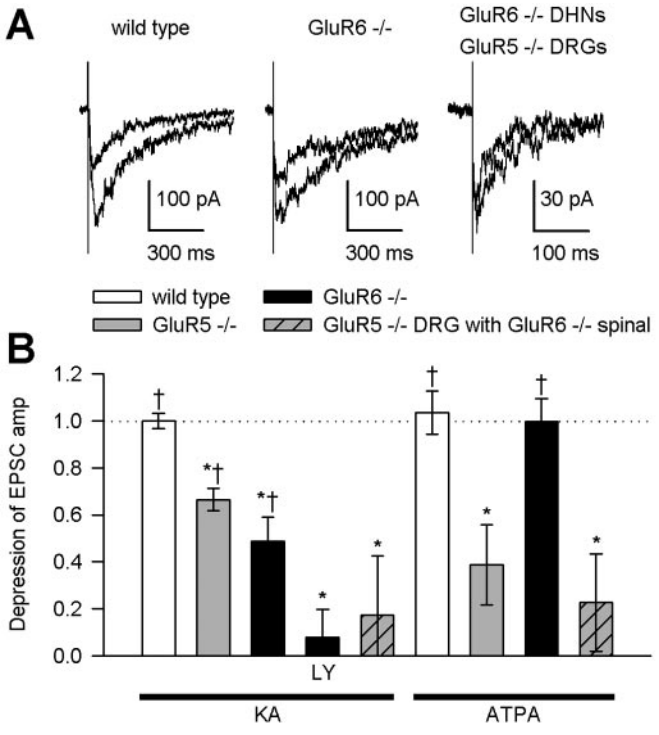

Figure 4. Roles of GluR5 and GluR6 in presynaptic KA receptor-mediated inhibition of dorsal horn excitatory transmission. *A*, Traces from representative experiments illustrate EPSCs in the absence (*bottom traces*) and presence (*top traces*) of 10 μM KA in cocultures comprised of neurons with the indicated genotypes. EPSCs were evoked in dorsal horn neurons (*DHNs*) by an extracellular stimulating electrode placed against the cell body of a nearby DRG cell. *B*, The effects of KA (10 μM) and ATPA (2 μM) on EPSC amplitude in DRG/spinal cocultures made from wild-type ($n = 4$ recordings in KA, 2 in ATPA), *GluR5−/−* ($n = 5$ KA, 3 ATPA), or *GluR6−/−* ($n = 11$ KA, 5 ATPA) mice plotted relative to the magnitude of KA action in wild-type cocultures. In some experiments, LY382884 (*LY*; 10 μM) was present continuously ($n = 3$). Some cocultures contained *GluR5−/−* DRG neurons and *GluR6−/−* dorsal horn neurons ($n = 4$ KA, 2 ATPA). *Significantly different from KA action in wild-type cultures. †Significantly different from baseline responses in the absence of KA or ATPA. *Dotted line* indicates 100% of normalized depression in wild type.

DISCUSSION

The GluR5 and GluR6 subunits make distinct contributions to KA receptors expressed by DRG neurons and dorsal horn neurons. In DRG cells, ATPA produced strong, long-lived desensitization (Fig. 3) (Kerchner et al., 2001b), and functional KA receptors were eliminated by *GluR5* deletion. These data confirm that receptors in wild-type DRG cells must contain GluR5 (Mulle et al., 2000) and that the pharmacology of wild-type receptors is dominated by features associated with the GluR5 subunit. Although peak KA-evoked current densities in DRG neurons were not affected by *GluR6* deletion, the differences in desensitization kinetics between wild-type and *GluR6−/−* cells (Fig. 3*D,E*) suggest a possible contribution of GluR6 to KA receptors in DRG cells that has hitherto been discounted (Sommer et al., 1992; Swanson et al., 1998). Other KA receptor subunits may contribute as well (Partin et al., 1993; Petralia et al., 1994).

In contrast to DRG cells, the density of KA-evoked currents in dorsal horn neurons was unaffected by *GluR5* deletion but was significantly reduced in *GluR6*−/− neurons. Approximately one-half of the *GluR6*−/− cells recorded did not respond to KA; cells that did respond exhibited smaller current densities than wild-type or *GluR5*−/− cells, all of which were sensitive to KA (Fig. 1*B,D*). Consistent with the direct measurements of KA currents, the effects of presynaptic KA receptor activation were preserved in spinal neurons lacking *GluR5* but were diminished with *GluR6* deletion. Thus, the GluR6 subunit is clearly an important component of KA receptors in most dorsal horn neurons.

The present study focuses on the properties of cultured neurons. Although the possibility exists that subunit expression in culture might differ from that *in vivo*, our previous studies in rats (Kerchner et al., 2001a,b) have documented good agreement between cell culture and acute slice preparations in the properties of KA receptor-mediated regulation of transmission.

Dorsal horn neurons: evidence for GluR5 expression

Although deletion of *GluR5* did not affect the amplitude or time course of KA receptor-mediated currents in dorsal horn neurons, our pharmacological data provide evidence for GluR5 expression in a significant proportion of cells from wild-type and *GluR6*−/− mice. The GluR5-selective antagonists LY382884 and LY293558 produced a substantial block in all of the wild-type and *GluR6*−/− neurons tested but had virtually no effect in *GluR5*−/− cells (Fig. 1*C*), confirming the selectivity of these compounds at native receptors (Bleakman et al., 1996). Compounds selective for GluR5 had a somewhat larger effect on dorsal horn neurons from *GluR6*−/− animals than on cells from wild-type mice or rats. In previous work on spinal neurons from rats (Kerchner et al., 2001b; Wilding and Huettner, 2001), fewer than half of the cells responded to ATPA; in those cells the ATPA-evoked currents were small compared with currents elicited by KA. Similarly, in dorsal horn neurons from wild-type mice, ATPA-evoked currents were small in proportion to KA; likewise, ATPA produced only a modest increase in mIPSC frequency in wild-type cultures (Fig. 2*B*). In contrast, relative peak current amplitude triggered by ATPA in cells from *GluR6*−/− mice was substantial (more than half of that triggered by KA) (Fig. 1*D,E*). In addition, brief exposure to ATPA produced significant cross desensitization of spinal KA receptors only in *GluR6*−/− cells (Fig. 1*E*). ATPA reliably triggered the quantal release of GABA and glycine in *GluR6*−/− dorsal horn neurons (Fig. 2*A,B*), further supporting the expression of GluR5.

These data clearly suggest that GluR5 and GluR6 both contribute to KA receptor-mediated currents in dorsal horn neurons in mice; however, it is more difficult to determine whether individual receptors are heteromeric or whether there exist distinct populations of homomeric receptors. The ability of GluR5 antagonists to block instantaneous current at the onset of agonist exposure in wild-type cells indicates that most surface receptors were affected by the drug and are therefore likely to include a GluR5 subunit. This result, together with the evidence discussed above implicating GluR6 as a component in most wild-type KA receptors, suggests that many dorsal horn neurons express heteromeric receptors that include both GluR5 and GluR6. The relative lack of effectiveness of ATPA to activate responses or to cross desensitize KA responses in wild-type neurons is not necessarily inconsistent with this reasoning, if the effect of this agonist depends on the stoichiometry of GluR5 within a heteromeric complex (Vignes et al., 1998). In other words, dorsal horn neurons may express KA receptors with a low ratio of GluR5 to other subunits sufficient to confer sensitivity to LY382884 but not to ATPA. This hypothesis might explain the finding that *GluR5* deletion had little to no effect on overall KA receptor-mediated current density. It could also explain why ATPA appears more effective in *GluR6*−/− cells than in wild type, because *GluR6* deletion might increase GluR5 stoichiometry at the level of individual receptors.

In addition to GluR5 and GluR6, other subunits may contribute to KA receptors in dorsal horn neurons. Tölle et al. (1993), using *in situ* hybridization to map the mRNA distribution for all five KA receptor subunits in adult rats, observed a prominent expression of KA2 in the superficial dorsal horn and substantially lower expression of KA1. Weak but widespread labeling was also observed for the GluR7 subunit and, in significantly fewer cells, for GluR5 (Furuyama et al., 1993). Tölle et al. (1993) reported that GluR6 mRNA was undetectable by *in situ* labeling in adult rat spinal cord; however, a more recent developmental *in situ* hybridization study (Stegenga and Kalb, 2001) suggests that spinal KA receptor subunit expression, including expression of GluR6, may be significantly higher in newborn animals. Our physiological results from both mice (Figs. 1, 2) and rats (Kerchner et al., 2001b; Wilding and Huettner, 2001) clearly highlight an important role for the GluR6 subunit and a less prominent role for GluR5 in the assembly of functional somatodendritic and presynaptic terminal KA receptors in dorsal horn neurons. In preliminary reverse transcription PCR experiments, we detected strong expression of GluR6 in both cultured and freshly isolated newborn rat dorsal horn (J. E. Huettner, unpublished observations). In future work, it will be of interest to test for production of functional KA receptors in *GluR5*−/− × *GluR6*−/− double knock-out mice, as well as in mice deficient in other KA receptor subunits, when they become available.

Finally, the prevalence of KA receptor subunits may vary among different subpopulations of dorsal horn neurons. Some cells may express heteromeric receptors that include both GluR6 and GluR5, whereas other cells may express GluR6 without GluR5. If this were true, then *GluR6* deletion should abolish KA currents in some dorsal horn neurons but not in others; this was indeed the case (Fig. 1*D*). Also supporting this hypothesis is the indirect evidence that *GluR6* deletion in dorsal horn neurons apparently prevented KA-induced suppression of spinal-to-spinal excitatory transmission in mixed *GluR5*−/− DRG/*GluR6*−/− spinal cocultures (Fig. 4) (also see below), suggesting that GluR6 may be required for KA receptor expression by glutamatergic dorsal horn neurons. In contrast, presynaptic KA receptors on inhibitory dorsal horn neurons were eliminated by neither *GluR5* nor *GluR6* deletion (Fig. 2) (also see above), indicating that inhibitory neurons likely contain heteromeric receptors that include both subunits, either of which is sufficient for the production of functional receptors. Mulle et al. (2000) reached a similar conclusion concerning the subunit contribution to KA receptors in hippocampal CA1 inhibitory interneurons. They showed that deletion of either *GluR5* or *GluR6* alone was not sufficient to eliminate KA receptors; however, receptors were abolished by the combined deletion of both subunits (Mulle et al., 2000).

DRG neurons

Unlike dorsal horn neurons, functional KA receptors on DRG neurons exhibited an absolute requirement for GluR5. GluR5 may form homomeric receptors on some DRG cells (Swanson et al., 1998) or it may combine with GluR6, GluR7, KA1, or KA2

(Partin et al., 1993). However, in *GluR5*−/− DRG cells, KA receptor-mediated currents were not detected, indicating that other subunits, if present at all, did not contribute to functional receptors in the absence of GluR5.

The involvement of both GluR5 and GluR6 in the KA-induced inhibition of excitatory transmission (Fig. 4) likely reflects activation by the extracellular stimulating electrode of presynaptic elements derived from both DRG and dorsal horn neurons. Even when the electrode is placed against the cell body of a DRG cell, the electrical field generated by the stimulating pulse may extend to include nearby dorsal horn neuronal cell bodies or axons; thus, excitatory dorsal horn neurons could contribute to a composite NMDA receptor-mediated EPSC. The slow kinetics of these EPSCs (Fig. 4*A*) would easily hide the presence of multiple responses with minor variations in latency. Supporting the notion that GluR5 is required for receptors regulating DRG to spinal transmission and that GluR6 is important for spinal-to-spinal excitatory transmission, neither KA nor ATPA affected EPSCs in mixed cocultures containing *GluR5*−/− DRG cells and *GluR6*−/− dorsal horn neurons (Fig. 4).

Although our results indicate that many DRG cells and spinal neurons are likely to express heteromeric KA receptors that include both the GluR5 and GluR6 subunits, we also confirmed in wild-type and subunit-deficient mice the profound difference in cross desensitization of KA receptors by ATPA between DRG cells and dorsal horn neurons. Additional experiments will be needed to determine whether this difference in pharmacology reflects differential KA receptor subunit expression or stoichiometry, expression of alternative splice variants of GluR5 and/or GluR6, or different interactions with cytoplasmic proteins or enzymes that may be unique to DRG or spinal cells. A molecular distinction between KA receptors on DRG neurons and those on dorsal horn neurons, underlying the potential for pharmacological selectivity, is particularly attractive from a clinical perspective.

It is predicted that selective manipulation of presynaptic KA receptors at primary afferent synapses would alter pain transmission with fewer side effects than might be apparent using nonselective agents. Consistent with the ability of ATPA to inhibit DRG to spinal transmission, some evidence already suggests that in rats, selective activation of GluR5-containing KA receptors reduces nociceptive spinal reflexes *in vitro* (Procter et al., 1998) and nociceptive behavioral responses *in vivo* (Mascias et al., 2001). Additional *in vivo* studies have shown that systemic administration of GluR5-selective antagonists reduces hyperalgesia (Sang et al., 1998; Simmons et al., 1998), implicating GluR5-containing receptors in nociceptive processing more generally. Although the location of receptors responsible for these behavioral effects remains to be established, these studies highlight GluR5-containing KA receptors as a possible therapeutic target. Elucidation of the pathways underlying these effects, and the development and testing of agents selective for other KA receptor subunits, represent important areas for future work.

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