

# Splice-Variant- and Stage-Specific RNA Editing of the *Drosophila* GABA Receptor Modulates Agonist Potency

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The molecular diversity of many gene products functioning in the nervous system is enhanced by alternative splicing and adenosine-to-inosine editing of pre-mRNA. Using RDL, a *Drosophila melanogaster* GABA-gated ion channel, we examined the functional impact of RNA editing at several sites along with alternative splicing of more than one exon. We show that alternative splicing and RNA editing have a combined influence on the potency of the neurotransmitter GABA, and the editing isoforms detected *in vivo* span the entire functional range of potencies seen for all possible edit variants expressed in *Xenopus laevis* oocytes. The extent of RNA editing is developmentally regulated and can also be linked to the choice of alternative exons. These results provide insights into how the rich diversity of signaling necessary for complex brain function can be achieved by relatively few genes.

## Introduction

Alternative splicing (Stetefeld and Ruegg, 2005) and adenosine (A)-to-inosine (I) editing of pre-mRNA (Keegan et al., 2001) lead to the generation from a single gene of multiple protein isoforms and may contribute to achieving complexity in highly evolved organisms that have a surprisingly low total gene count (Hahn and Wray, 2002). Whereas alternative splicing introduces, excises, or substitutes peptide regions of a protein, A-to-I editing alters individual nucleotides through adenosine deaminases acting on RNA (ADARs) (Keegan et al., 2004) converting select adenosine residues in pre-mRNA to inosine. Because inosine is interpreted by cellular machineries as guanosine (G), A-to-I editing generates transcripts with a nucleotide composition distinct from the corresponding genomic DNA.

Many neuronal gene products are diversified by alternative splicing (Li et al., 2007), defects of which are implicated in neurological diseases (Licatalosi and Darnell, 2006). The genome recoding by RNA editing occurs particularly in proteins involved in neuronal signaling (Hoopengardner et al., 2003), which is consistent with the neurological phenotypes observed for ADAR-deficient *Caenorhabditis elegans* (Tonkin et al., 2002), *Drosophila melanogaster* (Palladino et al., 2000), and mice (Higuchi et al., 2000). Ligand-gated ion channels (LGICs) play central roles in mediating neuronal signaling, and an increasing number of

LGICs are found to undergo RNA A-to-I editing which alters amino acid residues in functionally significant regions (Hoopengardner et al., 2003). The association of deficient RNA editing of the glutamate AMPA receptor subunit GluR2 with amyotrophic lateral sclerosis highlights the importance of RNA editing of LGICs (Kawahara et al., 2004).

Gene products can undergo both alternative splicing and RNA editing, and several reports of these processes being coregulated have been published (Laurencikiene et al., 2006; Schoft et al., 2007). For example, RNA editing leading to an arginine-to-glycine switch may regulate alternative splicing which generates Flip and Flop variants in glutamate receptors (Schoft et al., 2007). RNA editing can also alter several nucleotides of a transcript without affecting splicing. However, the combined functional effects of such editing and multiple alternative splicing events of LGICs remain to be determined. We have, therefore, addressed this question using RDL, a homomer-forming GABA receptor subunit (Hosie et al., 1997). RDL mediates fast synaptic inhibition in the nervous system of *D. melanogaster*, where it is an important modulator of olfactory associative learning (Liu et al., 2007) and sleep latency (Agosto et al., 2008). Splicing of *Rdl* yields two variants each for exons 3 (variants a and b) and 6 (variants c and d) (Buckingham et al., 2005) (Fig. 1), and all four possible alternative isoforms (ac, ad, bc, and bd) are transcribed (French-Constant and Rocheleau, 1993). In addition, *Rdl* undergoes A-to-I editing (Hoopengardner et al., 2003), which leads to the substitution of four amino acid residues. Thus, the combination of alternative splicing and RNA editing has the potential to generate considerable variation in the RDL receptor.

Here, we report an analysis of all possible editing combinations in two splice variant backgrounds. We show that editing and the choice of alternative exons are linked and that both processes act in concert to alter GABA potency suggesting a key role for both processes in fine-tuning neuronal signaling.

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## Materials and Methods

*Drosophila stocks.* Oregon-R flies were used in this study.

*RNA editing analysis.* Embryo total RNA was obtained from Stratagene UK, and total RNA was extracted from CNSs dissected from L3 larvae or adult (mixed gender) using Trizol (Invitrogen). cDNA was synthesized from the total RNA using Expand reverse transcriptase (Roche) and an oligo(dT)<sub>18</sub> primer to generate template for PCR using *Pfu* Turbo DNA polymerase (Stratagene). The extent of RNA editing shown in Figure 2 was estimated by ratiometric A/G measurement from sequence chromatograms (Jepson and Reenan, 2007) of PCR products amplified using primers specific to alternative exons (supplemental Fig. 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). To determine RNA editing profiles shown in Figure 3, *Rdl* PCR products amplified from adult CNS using *Pfu* Turbo DNA polymerase (Stratagene), and primers listed in supplemental Figure 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material, were cloned into pCR-Blunt (Invitrogen) and sequenced.

*SYBR Green PCR.* To determine relative transcript levels of *Rdl* splice variants, SYBR Green PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7000 Sequence Detection System. Primers listed in supplemental Figure 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material, were used on adult CNS cDNA template. The comparative cycle threshold method (User Bulletin 2, 1997; Applied Biosystems) was used to calculate relative values of the *Rdl* splice variant cDNA normalized against the calibrator gene *rp49*.

*Two-electrode voltage-clamp electrophysiology.* The vectors pNB14.1 encoding *Rdl<sub>ac</sub>* (Hosie et al., 2001) or *Rdl<sub>bc</sub>* (constructed in this study) as well as pcDNA3.1/Zeo+ (Invitrogen) with *Rdl<sub>bd</sub>* (constructed in this study) were used. Point mutations corresponding to all possible combinations of edited and unedited residues were generated in *Rdl<sub>ac</sub>* and *Rdl<sub>bd</sub>* splice variants using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). pNB14.1 *Rdl<sub>ac</sub>* or *Rdl<sub>bc</sub>* and pcDNA3.1/Zeo+ *Rdl<sub>bd</sub>* were linearized to be used as template for cRNA synthesis with the SP6 and T7 mMESSAGE mMACHINE kits (Ambion), respectively. *Xenopus laevis* oocytes, which were injected with combinations of cRNAs to a total of 50 nl cRNA (1 ng/ml), were incubated and voltage clamped at  $-60$  mV 2–4 d after injection as described previously (Hosie et al., 2001; Buckingham et al., 2006). Concentration–response curves were constructed by challenging oocytes with increasing concentrations of GABA at a flow rate of 5 ml/min and with at least 2 min between challenges to reduce the effects of desensitization. Peak current amplitudes were measured as the maximum deflection of the current trace from the baseline using Clampfit v8.1. EC<sub>50</sub>s were determined by normalizing the concentration–response curve obtained from each oocyte to the maximum current obtained from that oocyte and fitting the normalized data to the following expression:

$$Y = \frac{1}{1 + 10^{(X_{50} - X)_{nH}}}$$

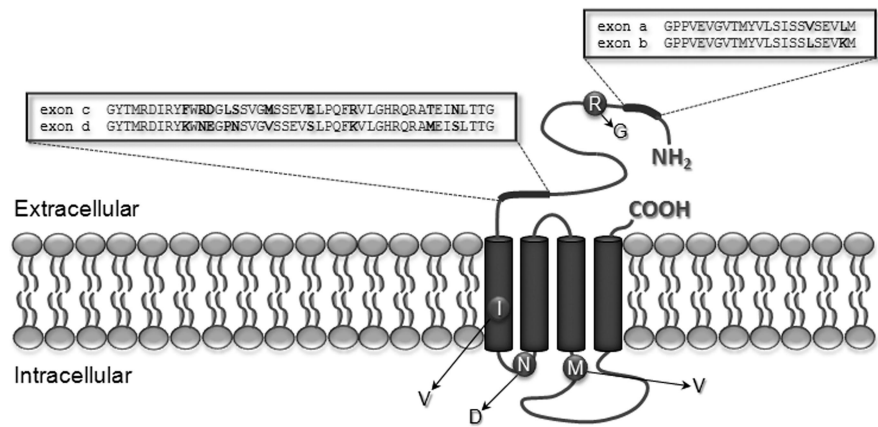
where  $Y$  is the normalized response amplitude,  $X_{50}$  is the log<sub>10</sub> of the EC<sub>50</sub>,  $X$  is the log<sub>10</sub> of the GABA concentration (M), and  $nH$  is the Hill coefficient. Fits to the data were performed using GraphPad Prism.

*Statistical analysis.* Data were analyzed by either one-way or two-way ANOVA followed, if significant, by the Tukey post-test using GraphPad Prism 4.

## Results

### RNA editing of *Rdl* is developmentally regulated and can be dependent on the choice of alternative exons

We analyzed sequence traces of cDNA PCR products to investigate the extent of RNA editing in each of the splice variants at

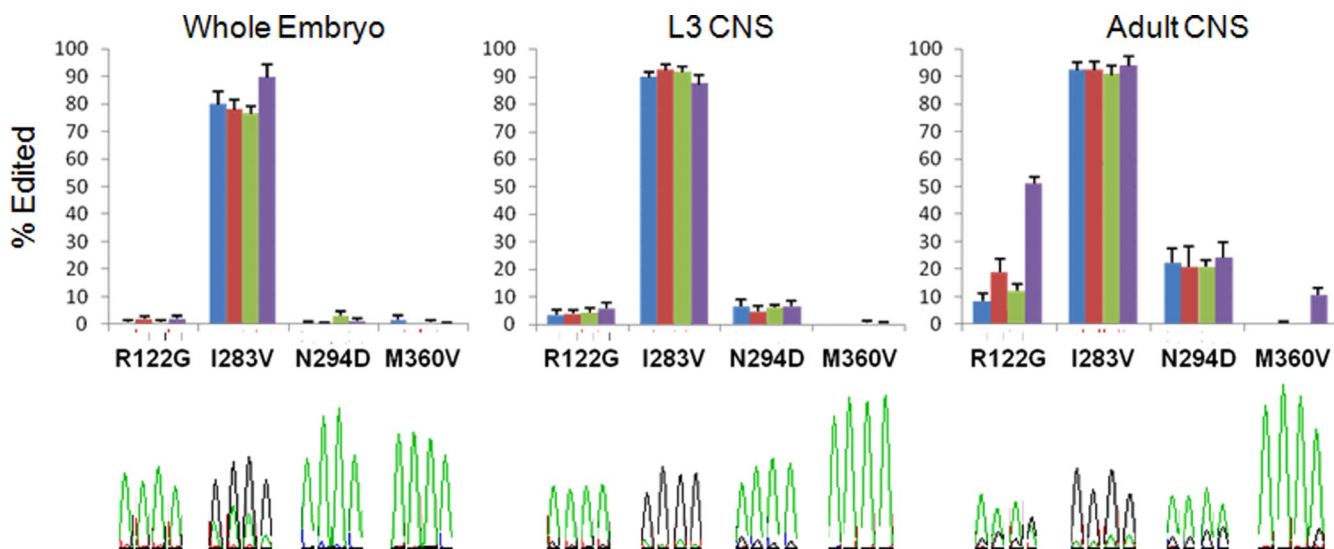


**Figure 1.** Schematic illustration showing alternative splicing and RNA editing in the *Rdl* subunit. Alternative splicing of *Rdl* yields two variants each for exons 3 (variants a and b) and 6 (variants c and d) changing amino acid sequence in the N-terminal extracellular domain where ligand binding occurs. Residues that are different in alternative exons are shown in bold. RNA editing leads to the substitution of arginine to glycine (R122G) in the N-terminal extracellular domain and of isoleucine to valine (I283V) in the first transmembrane domain, whereas asparagine and methionine residues in intracellular regions are converted to aspartic acid (N294D) and valine (M360V), respectively.

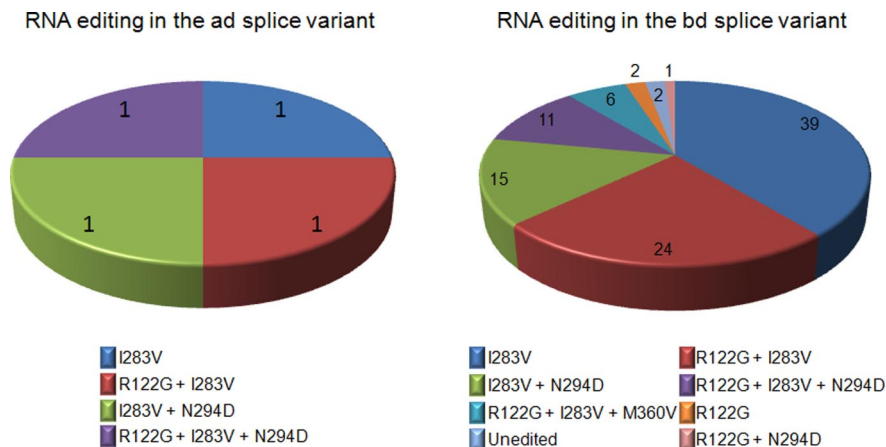
three stages of *Drosophila* development (whole embryo, larval L3, and adult nervous system). Consistent with findings that RNA A-to-I editing is primarily involved in the adult nervous system (Palladino et al., 2000), it was here that we found the greatest extent of RNA editing (Fig. 2). Our analysis also revealed that the implementation of editing at each site varied with developmental stage. For example, we found only I283V editing in the embryo, whereas R122G and N294D editing was also detected in larval nervous system, and M360V editing was seen only in adults. Editing levels vary between each site, as shown by our discovery that isoleucine is predominantly edited, whereas the other three sites are mostly unedited. When considering different splice variants of a given edit site, editing levels can be either similar (e.g., I283V or N294D in the adult nervous system) or different, as shown by significantly higher R122G editing levels in the bd splice variant ( $p < 0.001$ ). The inclusion of certain alternative exons and the decision to edit might in some cases be linked, as we saw M360V editing only in the bd splice variant. Thus, the extent of RNA editing is dependent on developmental stage and can differ between alternative splice variants.

### Particular editing profiles are favored *in vivo*

To determine RNA editing profiles present *in vivo*, we sequenced cloned *Rdl* subunits amplified from adult nervous system cDNA. Of the 104 clones analyzed, four were of the ad splice isoform, whereas the remaining 100 were bd. SYBR Green PCR, using primers shown in supplemental Figure 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material, was used to confirm that the bd isoform is clearly the predominant splice variant, with transcript levels 26 times greater than ad, which is the second most abundant variant (Table 1). Each of the four ad clones has a different combination of editing, which corresponds to the four most abundant isoforms seen in the bd transcripts (Fig. 3). As shown in Figure 3, only half of the 16 possible isoforms that could arise from RNA editing were detected in the bd clones, indicating that certain edit isoforms are preferentially adopted and that editing at distinct sites is linked. Editing combinations in sequenced clones were present in varying proportions ranging from 1 to 39%. A small proportion (2%) of sequenced subunits was completely unedited, and fully edited isoforms were not detected.



**Figure 2.** The extent of RNA editing in *Rdl* is related to developmental stage and alternative splicing. Percentage editing at the four sites (R122G, I283V, N294D, and M360V) is shown for each of the four possible splice variants, ac (blue), ad (red), bc (green), and bd (purple), at three developmental stages of *Drosophila* (whole embryo, L3 CNS, and adult CNS). Values are shown as mean + SEM ( $n = 5$ ). Also shown are example traces from cDNA sequence chromatograms corresponding to each of the editing sites in the four splice variants. The presence of black peaks (guanosine) with the green peaks (adenosine) indicates RNA A-to-I editing.



**Figure 3.** Particular RNA editing profiles are highly represented *in vivo*. The pie charts show the RNA editing isoforms found in the 104 clones analyzed. Four of the clones have ad alternative exons, whereas the remaining 100 have bd.

**Table 1. *Rdl* splice variants show differential transcript levels and GABA potency**

Splice variant	Transcript levels relative to <i>Rdl<sub>ac</sub></i>	GABA EC <sub>50</sub> ( $\mu$ M)
ac	1 ± 0.5	27 ± 1*
ad	11.9 ± 2.8	58 ± 4*
bc	7.5 ± 1.6	20 ± 0.2
bd	311 ± 1.1	152 ± 10*

Relative transcript levels are given as mean ± SEM ( $n = 3$ ). GABA EC<sub>50</sub> values marked by an asterisk are previously published (Hosie et al., 2001), while the concentration–response curve for RDL<sub>bc</sub> is shown in supplemental Figure 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material. Note that all splice variants used to generate the EC<sub>50</sub>s have I283V editing. EC<sub>50</sub>s are shown as mean ± SEM ( $n \geq 3$ ).

M360V editing, which was only seen in the bd splice variant (Fig. 2), was observed only in combination with R122G and I283V editing. In contrast, R122G, I283V, and N294D editing was seen in various combinations. The proportion of editing at each site of bd transcripts shows a reasonable correlation with editing levels estimated by measuring A/G peak height ratios (Table 2) showing a consistency between two techniques used to quantify RNA editing (Jepson and Reenan, 2007).

### Alternative splicing of *Rdl* affects GABA potency

Previous work with three RDL variants (ac, ad, and bd) has shown that alternative splicing affects agonist potency (Hosie et al., 2001). Using the two-electrode voltage-clamp technique (Buckingham et al., 2006), we report the GABA EC<sub>50</sub> for the fourth splice variant, bc, which is similar to that of RDL<sub>ac</sub> (Table 1; supplemental Fig. 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). RDL<sub>bd</sub> shows the most strikingly different EC<sub>50</sub> indicating that GABA acts with less potency on this isoform than the other three splice variants. Interestingly, RDL<sub>bd</sub> is distinct in other respects because we found that when compared with other splice variants, bd transcripts are by far the most abundant

(Table 1) and that bd is the only splice variant observed to be edited at all four sites (Fig. 2).

### Alternative splicing and RNA editing have a combined effect on GABA potency

To study the impact of alternative splicing and RNA editing of a ligand-gated ion channel on agonist potency, we introduced point mutations to generate isoforms reflecting all 16 possible combinations of nonediting and editing in both *Rdl<sub>ac</sub>* and *Rdl<sub>bd</sub>*. These two splice variants cover all four alternative exons and are also the least and most abundant, respectively, of the four splice isoforms (Table 1). Capped RNA of the 32 variants were injected into *Xenopus laevis* oocytes for functional analysis. Application of GABA (1  $\mu$ M–3 mM) to oocytes expressing *Rdl* resulted in concentration-dependent inward currents for all editing isoforms in both splice backgrounds tested. The relatively slow delivery of GABA in the *Xenopus* oocyte expression system does not permit the accurate measurement of current kinetics. Nevertheless, oocytes give highly robust and reproducible responses, and the expression of insect LGICs in other systems have often proved



**Table 2. Editing levels obtained by clone counting (Fig. 3) are consistent with levels determined by measuring A/G peak height ratios (Fig. 2)**

Edit site	A/G peak height ratio	Clone counting
R122G	51 ± 2.1	44
I283V	94 ± 3.4	95
N294D	24 ± 5.4	27
M360V	10 ± 2.5	6

Values are indicated as percentages edited in the *Rdl*<sub>bd</sub> splice variant from adult CNS. A/G peak height ratios are shown as mean ± SEM ( $n = 5$ ).

difficult. We determined the GABA EC<sub>50</sub>s which varied up to 60-fold between different edit and splice variants (range, 3.2–192.9 μM) (Fig. 4, Table 3). Responses to high concentrations of GABA relative to the EC<sub>50</sub> declined in amplitude, as evidenced by a fall in  $I/I_{max}$  at the right hand of the concentration response curves (Fig. 4*b*) (RDL<sub>bd</sub> I283v plus N294D). This is likely attributable to the large current fluxes involved, because such large responses were accompanied by a change in reversal potential (data not shown).

Two-way ANOVA for the effects of editing and splicing on the EC<sub>50</sub> using unweighted means indicated that effects of editing and splicing on EC<sub>50</sub>s were both highly significant ( $p < 0.0001$ ). In addition, there was a significant interaction between editing and splicing ( $p < 0.0001$ ), indicating that the effects of editing these residues differs between the two splice backgrounds. The ANOVA also indicated that splicing contributed an estimated 38% percent to the variance, with editing contributing 19% and the interaction between editing and splicing accounting for 16%. In one striking example, I283V plus N294D and R122G plus I283V plus N294D edit isoforms in the bd splice variant showed significantly different ( $p < 0.001$ ) GABA EC<sub>50</sub>s of 27.4 μM and 192.9 μM, respectively (Fig. 4*b,c*, Table 3), which are the lowest and highest values among all of the possible edit isoforms. For the ac splice variant, this is not the case as the GABA EC<sub>50</sub>s of these two edit isoforms are similar with 3.2 and 4.2 μM (Table 3). Thus, editing and splicing can have a combined effect on agonist potency. Interestingly, the EC<sub>50</sub>s of the splice/edit forms identified *in vivo* (Fig. 3) span the whole range of values including the two extremes (Fig. 4*c*), suggesting that RNA editing adds precision modulation to the function of proteins involved in rapid neurotransmission.

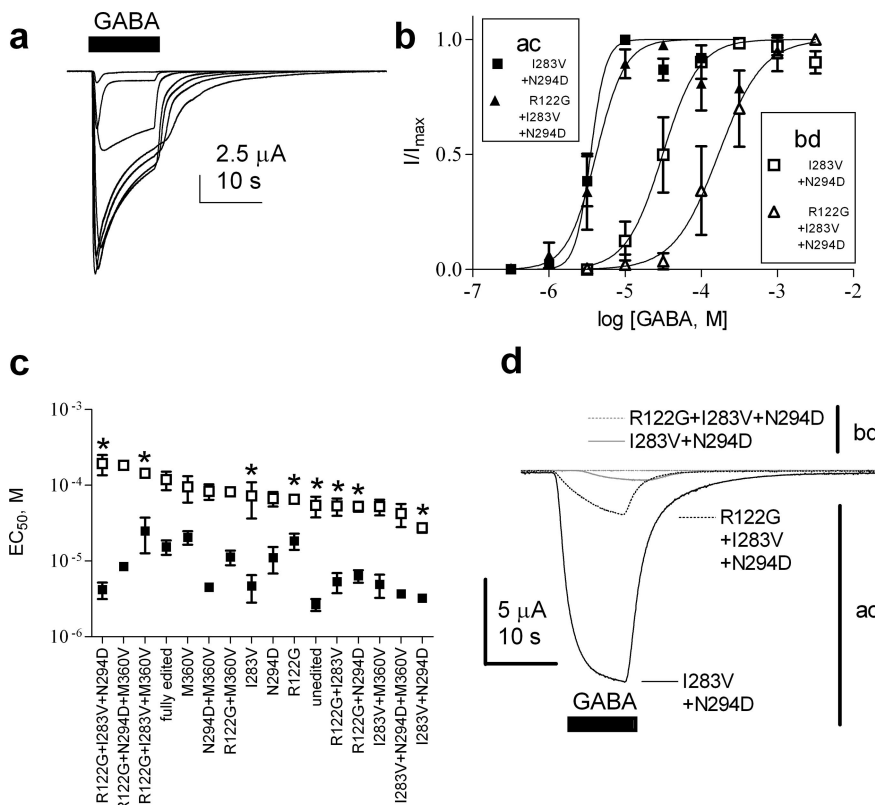
Two-way ANOVA using unweighted means showed that alternative splicing had a significant effect on the maximum currents ( $p < 0.0001$ ) (supplemental Table 1, available at www.jneurosci.org as supplemental material), whereas RNA editing did not. This is highlighted in Figure 4*d*, where I283V plus N294D and R122G plus I283V plus N294D edit isoforms in the ac and bd splice variants show considerably different current amplitudes in response to the same GABA concentration. The different currents indicate that alternative splicing of *Rdl* may influence receptor trafficking, assembly, and/or channel conductance or kinetics.

## Discussion

The diversity of LGICs can be broadened considerably by the presence of more than one alternatively spliced exon along with RNA editing at multiple sites. To investigate the functional consequences of such complexity, we expressed in *Xenopus* oocytes all 16 possible editing isoforms of *Rdl* in two splice variants and measured their effect on GABA potency. Our study is the first functional analysis encompassing both RNA editing and alternative splicing in a cys-loop LGIC, which is a member of a superfamily of important signaling molecules including nicotinic ace-

tylcholine receptors (Kalamida et al., 2007), serotonin type 3 receptors (Reeves and Lummis, 2002), and GABA receptors (Bormann, 2000). Previous studies have shown that alternative splicing of *Rdl* affects agonist potency (Hosie et al., 2001). In line with this, splice variants have different amino acids in functionally significant regions. Thus, alternative splicing of exon 3 effectively introduces amino acid diversity near a highly conserved valine, which is proposed to form a contact between the ligand-binding and transmembrane domains (Miyazawa et al., 2003), suggesting that *Rdl* exon 3 is involved in the coupling of ligand binding to pore opening (Buckingham et al., 2005). Alternative splicing of exon 6 is likely to directly affect GABA potency, because it generates diversity in loops C and F which contribute to the ligand-binding site (Buckingham et al., 2005). As with exon 6 splicing (Hosie et al., 2001), R122G RNA editing may also directly affect agonist potency, because it alters a residue located between ligand-binding loops D and A. This was shown to be the case where the replacement of arginine by glycine slightly but significantly increases GABA EC<sub>50</sub> in the bd splice variant (Es-Salah et al., 2008). In our study, we also found that R122G, when compared with the unedited isoform, shows a slightly increased EC<sub>50</sub> for GABA (Table 3), although our analysis considering all bd edit isoforms indicated this increase was not significantly different. However, R122G editing in the bd splice variant had a dramatic effect on GABA potency when in combination with I283V plus N294D editing, because R122G plus I283V plus N294D and I283V plus N294D have significantly different ( $p < 0.001$ ) EC<sub>50</sub>s (Table 3). I283 and N294 are located, respectively, in the intracellular end of TM1 and the linker between TM1 and TM2 (Fig. 1), both of which regions are proposed to contribute to the pathway of the permeating ion (Filippova et al., 2004). Substitution of the equivalent residues with cysteine in the human  $\rho 1$  GABA subunit significantly alters GABA EC<sub>50</sub> (Filippova et al., 2004). We found that individually, or in combination, I283V and N294D editing had no significant effect on RDL<sub>bd</sub> GABA EC<sub>50</sub> (Table 3), although the triple edit isoform R122G plus I283V plus N294D had a significantly different GABA EC<sub>50</sub> to both R122G plus I283V and R122G plus N294D ( $p < 0.01$ ) (Table 3), indicating that I283V and N294D editing influences GABA sensitivity when in combination with editing at other sites. The finding that R122G, I283V, and N294D editing alone or as double edit (any two from three) isoforms has no significant effect on GABA EC<sub>50</sub> (Table 3) further points to a combinatorial effect of RNA editing at several sites which is relevant *in vivo*, because we sequenced clones with almost all of these editing profiles (Fig. 3). The combinatorial effect of the R122G plus I283V plus N294D triple edit isoform seen in bd was not mirrored in the ac splice variant (Table 3, Fig. 4*b*), showing that alternative splicing presents another factor in determining the effectiveness of RNA editing in modulating agonist potency. Our results, therefore, highlight that RNA editing at several sites can act in concert and with alternative splicing to broaden, considerably, the spectrum of LGIC sensitivity to its neurotransmitter.

M360V editing *in vivo* was only seen in the bd splice variant (Fig. 2) and only in combination with R122G and I283V editing (Fig. 3), indicating that particular RNA editing profiles are preferentially adopted in certain splice variants. M360 is located in the large intracellular loop between TM3 and TM4 (Fig. 1), which has been shown to influence GABA<sub>A</sub> desensitization kinetics and GABA EC<sub>50</sub> (Fisher, 2004). We found that the EC<sub>50</sub> of R122G plus I283V plus M360V is among the highest of all possible RDL<sub>bd</sub> edit isoforms (Table 3, Fig. 4*c*). As can be seen in Figure 4*c*, the EC<sub>50</sub> of R122G plus I283V plus M360V, along with the other seven edit



**Figure 4.** Alternative splicing and RNA editing of *Rdl* affect GABA potency. *a*, Representative current responses of RDL expressed in *Xenopus* oocytes to GABA at concentrations from 1  $\mu$ M to 3 mM. *b*, Example concentration–response curves of two RDL edit isoforms in ac and bd splice backgrounds. *c*, The graph shows GABA  $EC_{50}$  values of all possible editing isoforms as mean  $\pm$  SEM ( $n = 3–7$  oocytes, each oocyte from a different animal), ordered from left to right according to the  $EC_{50}$  values for the bd splice variant. All the actual  $EC_{50}$  values are listed in Table 3, and the two concentration–response curves giving the extreme  $EC_{50}$  values in the bd splice variant are shown in Figure 4*b*.  $EC_{50}$  values of edit isoforms found to exist *in vivo* through clone analysis (Fig. 3) are marked by an asterisk. Closed squares, ac variant; open squares, bd variant. *d*, Splice/edit isoforms can show variation in current response to the same GABA concentration (4 mM).

**Table 3.** The  $EC_{50}$  values for all the splicing and editing combinations performed in the present study

	ac			bd		
	$EC_{50}$ , $\mu$ M	SEM	<i>n</i>	$EC_{50}$ , $\mu$ M	SEM	<i>n</i>
R122G plus I283V plus N294D	4.2	1	4	192.9	57.3	4
R122G plus N294D plus M360V	8.5	0.8	4	182.8	19.2	4
R122G plus I283V plus M360V	24.9	12.3	5	143.5	13.4	4
Fully edited	15.4	3.3	5	118.6	32.2	7
M360V	20.6	4.2	5	95	36.1	3
N294D plus M360V	4.5	0.6	5	83.6	19.4	4
R122G plus M360V	11.3	2.5	5	81.8	8.4	5
I283V	4.7	1.9	5	72.6	36.5	4
N294D	11.1	4.3	5	66.9	14.7	4
R122G	18.5	4.5	3	65.6	7.4	4
Unedited	2.7	0.5	5	54	16.6	4
R122G plus I283V	5.3	1.6	5	53.2	13.8	5
R122G plus N294D	6.4	1.2	4	52.6	8.2	6
I283V plus M360V	4.9	1.7	4	52.3	12.2	3
I283V plus N294D plus M360V	3.7	0.4	5	42.3	14.4	5
I283V plus N294D	3.2	0.2	5	27.4	3.9	6

The SEM and number of experiments from different batches of oocytes are also given. The  $EC_{50}$  values are arranged in descending order for the bd splice variant.

isoforms observed *in vivo*, span the entire functional range seen for all possible edit isoforms, suggesting a role for RNA editing in fine-tuning LGIC function.

In addition to its actions on  $EC_{50}$ , we also noted that splicing affected the maximum amplitude of GABA-evoked currents (Fig.

4*d*, supplemental Table 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). This may reflect changes in the receptors, such as alterations in single-channel conductance or open time, or it could point to changes in the efficiency of receptor trafficking or assembly. Thus, in concert, alternative splicing and RNA A-to-I editing have the potential to profoundly influence GABA-mediated inhibitory signaling in the insect CNS.

It has been suggested that in glutamate receptors, arginine-to-glycine editing influences Flip Flop alternative splicing, because the edit site is located only one nucleotide away from the 5' splice site of intron 13 (Barbon et al., 2003; Schoft et al., 2007). Editing could, thereby, reduce the efficiency of a 5' consensus splice site or introduce a splice silencer consensus sequence. A similar process is unlikely to account for the links between alternative splicing and RNA editing we observed for *Rdl*, because the four editing sites are not located adjacent to exon boundaries. The mechanism of RNA editing involves ADARs targeting the site to be edited which has formed a duplex by base pairing with a complementary RNA strand known as the editing site complementary sequence (ECS) (Jepson and Reenan, 2007). The ECS, which is commonly located in the intron downstream of the exon containing the editing site, forms a hairpin structure with the editing site (Hanrahan et al., 2000), although a more complex pseudoknot structure has been described where intronic elements pair with separate exonic regions to create two ADAR target sites (Reenan, 2005). Our findings suggesting that alternative splicing of exons is coordinated with preferential editing at several sites may add to the complexity of RNA structures recognized by ADARs.

Thus, the RDL receptor should be thought of in terms of a rich array of splice and edit variants. Interestingly, a study on *Drosophila* neurons demonstrated heterogeneity in GABA receptors containing RDL (Lee et al., 2003), which, in light of this report, may be due to the differential expression of various splice/edit isoforms. A future challenge, therefore, is to determine the precise stoichiometry of subunit isoforms which make up native RDL receptors *in vivo*. Similar or even greater complexity may be achieved in vertebrate GABA-gated ion channels because there

are more subunit-encoding genes, several products of which are alternatively spliced (Whiting et al., 1990; Poulsen et al., 2000; Tian et al., 2005), and, recently, A-to-I editing has been shown to modulate function of the GABA $_A$   $\alpha 3$  subunit (Ohlson et al., 2007; Rula et al., 2008). By examining all possible edit combina-

tions in the most abundant and least abundant splice variants, we have uncovered a profound repertoire of neurotransmitter responses with major implications for timing in inhibitory circuits and the consequent impact on behavior.

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