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Furthering pharmacological and physiological assessment of the glutamatergic receptors at the *Drosophila* neuromuscular junction

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ABSTRACT

Drosophila melanogaster larval neuromuscular junctions (NMJs) serve as a model for synaptic physiology. The molecular sequences of the postsynaptic glutamate receptors have been described; however, the pharmacological profile has not been fully elucidated. The postsynaptic molecular sequence suggests a novel glutamate receptor subtype. Kainate does not depolarize the muscle, but dampens evoked EPSP amplitudes. Quantal responses show a decreased amplitude and area under the voltage curve indicative of reduced postsynaptic receptor sensitivity to glutamate transmission. ATPA, a kainate receptor agonist, did not mimic kainate's action. The metabotropic glutamate receptor agonist t-ACPD had no effect. Domoic acid, a kainate/AMPA receptor agonist, blocks the postsynaptic receptors without depolarizing the muscle. However, SYM 2081, a kainate receptor agonist, did depolarize the muscle and reduce the EPSP amplitude at 1 mM but not at 0.1 mM. This supports the notion that these are generally a quisqualate subtype receptors with some oddities in the pharmacological profile. The results suggest a direct postsynaptic action of kainate due to partial antagonist action on the quisqualate receptors. There does not appear to be presynaptic auto-regulation via a kainate receptor subtype or a metabotropic auto-receptor. This study aids in furthering the pharmokinetic profiling and specificity of the receptor subtypes.

1. Introduction

The function and pharmacology of glutamate receptors in general are of prime interest since they are the major excitatory receptor type used within the mammalian central nervous system (CNS). In mammalian preparations, there are extensive investigations into the pharmacology, physiology, molecular structure and regulation of expression as well as turnover of glutamate receptors (Dingledine et al., 1999; Hollman and Hainemann, 1994; Lipsky and Goldman, 2003; Mayer, 2005; Pauly et al., 2005; Seeburg, 1993; Thiagarajan et al., 2005). Earlier studies on glutamate receptors at the neuromuscular junction of insects and crustaceans paved the way to dissecting the functional aspects of these receptors within vertebrates (Anderson et al., 1976; Cull-Candy and Parker, 1983; Gration et al., 1981; Jan and Jan, 1976; Patlak et al., 1979; Shinozaki and Ishida, 1981; Shinozaki and Shibuya, 1974). Still today, invertebrate models serve a vital role in describing the regulation and developmental aspects of glutamate receptor function (Featherstone et al., 2005; Guerrero et al., 2005; Littleton and Ganetzky, 2000; Logsdon et al., 2006; Pawlu et al., 2004; Rasse et al., 2005). Considering that *Drosophila melanogaster* is a true model organism with a known genome and used for successful rapid induction of mutations in various studies of development and synaptic mechanisms, it is important to understand the pharmacological and physiological function within this model synaptic neuromuscular preparation (Atwood et al., 1993; Betz et al., 1993; Jan and Jan 1976; Kurdyak et al., 1994; Li et al., 2002; Li and Cooper, 2001; Pawlu et al., 2004; Ruffner et al., 1999; Sigrist et al., 2002, 2003; Stewart et al., 1994, 1996).

The molecular sequence of the glutamate receptors on the *Drosophila* skeletal muscle has been described; however, to date there are few detailed investigations on the pharmacological profile of the intact larval *Drosophila* NMJ (Chang and Kidokoro, 1996; Chang et al., 1994; Delgado et al., 1989; Schuster et al., 1991; Zhang et al., 1999). Glutamate receptors are defined as NMDA (N-methyl-D-aspartic acid) and non-NMDA subtypes (i.e., AMPA, kainate and δ) based on their pharmacological sensitivity. The molecular sequences of the glutamate receptors for *Drosophila* NMJs were performed on the body wall muscles in larvae by isolating the skeletal muscle and the body wall (skeletal muscles attach to the cuticle body wall) (Qin et al., 2005). The total mRNA was extracted from this muscle tissue and probed

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against an "affymetrix *Drosophila* genechip". A number of *Drosophila* glutamate receptor subunits were found to be present. The sequences isolated were identified by comparison to whole larval DNA known sequences for glutamate receptors. The ones identified are GluRIIA, GluRIIB, GluRIIC and GluRIID. GluRIIE was later isolated when real-time PCR was used to follow up on the amplified mRNA of the four receptor subtypes in quantification of expression levels. The expression profile of the five glutamate receptor subunits has been conducted in body wall muscle by in situ hybridization for embryos and larvae (Guerrero et al., 2005; Marrus et al., 2004).

With the RT-PCR and the genomic profiling the sequence indicates that there are 5 glutamate receptor subunits (DGluR-IIA, DGluR-IIB, DGluR-IIC, DGluR-IID, DGluR-IIE) expressed in skeletal muscle and all of them are ionotropic receptors. When the molecular sequence of these 5 muscle-expressed glutamate receptors were compared with vertebrates, they showed the most similarity to the vertebrate kainate/AMPA receptor subtypes; therefore, they have been defined as a kainate receptor subtype without pharmacological examination (Betz et al., 1993; Guerrero et al., 2005; Marrus et al., 2004; Qin et al., 2005; Völkner et al., 2000). However, a recent physiological and pharmacological study demonstrates that the responses are primarily of a quisqualate subtype and not a kainate or a AMPA subtype since the muscle is not depolarized by kainate or AMPA at concentrations as high as 1 mM (Bhatt and Cooper, 2005). In fact, it appears that kainate might block the glutamate receptors since the excitatory postsynaptic potential (EPSP) is reduced in the presence of kainate (on average by 40% for 1 mM and 500 μM, but about 20% for 100 μM). The reduction in the EPSP amplitude occurred without any significant change in resting membrane potential; thus, kainate did not depolarize the muscle as observed for application of glutamate or quisqualate (Bhatt and Cooper, 2005).

In addition, the procedures used to identify the molecular sequence of the glutamate receptors at the NMJ, by homogenizing the skeletal muscle and body wall, did not allow for assessment for potential presynaptic glutamate receptors (Guerrero et al., 2005; Marrus et al., 2004; Qin et al., 2005). In the past procedure, the CNS was removed and thus the cell bodies of the motor neurons were not incorporated in the assay. Considering that there are presynaptic glutamatergic receptors for neurons in the vertebrate CNS (Park et al., 2006) and at crayfish NMJs (Schramm and Dudel, 1997), there may well be pre-synaptic autoreceptors present at the *Drosophila* NMJ. Crayfish NMJs show many similarities to the larval *Drosophila* NMJs (Atwood and Cooper, 1995,1996) which is not so surprising since both crayfish and *Drosophila* are arthropods. However, mixed results for the glutamatergic autoreceptors were obtained at the crayfish NMJ in that the responses depended on the depolarized state of the nerve terminal for the facilitatory action of exogenously applied glutamate and for the metabotropic agonist t-ACPD (Schramm and Dudel, 1997). In addition, some preparations showed a depression of presynaptic release while yet other preparations exhibited excitation at the same concentration of t-ACPD. Regardless of the response at the crayfish NMJ, there appears to be one or more types of presynaptic, glutamatergic receptors for the crayfish preparation (Schramm and Dudel, 1997).

The earlier observation of the rapid reduction in the EPSP by kainate at the *Drosophila* NMJ is most likely due to one or both of the following factors: (1) antagonist action on the postsynaptic glutamate receptors; (2) a reduction in the number of presynaptic vesicular fusion event. Direct blocking action on postsynaptic receptors is a possibility for kainate as many known agonists can be antagonists for subtypes within a receptor family (Chang and Weiss, 2002; Eberle et al., 2001; Hruby et al., 2003). This is likely, as a part of the extracellular receptor domains for glutamate receptors must be similar in order to bind glutamate. However, at the 3rd instar *Drosophila* NMJ the binding of kainate does not transduce activation of the glutamate ligand gated ion channels (Bhatt and Cooper, 2005; Dudel et al., 1992; Heckmann and Dudel, 1997). There is also precedence for kainate, a potent agonist for some vertebrate metabotropic receptors, to act presynaptically in reducing evoked transmitter release via autoreceptors on the presynaptic terminals (Chittajallu et al., 1996; Frerking et al., 2001; Kamiya and Ozawa, 1998; McLarnon and Quastel, 1988; Park et al., 2006). Kainate is known to bind metabotropic glutamate receptors (Zhang et al., 1999) and have a presynaptic action in altering transmitter release from presynaptic terminals in vertebrates (Kamiya and Ozawa, 2000; Kamiya et al., 2002; Park et al., 2006). In rats, kainate and the kainate receptor agonist ATPA, act presynaptically in reducing glutamate release from primary sensory neurons (Kerchner et al., 2001). However, in the rat neocortex, kainate receptors have been demonstrated to have both facilitatory as well as inhibitory actions due to dose dependent effect. The mechanism proposed for the biphasic effect is that low doses excite the presynaptic terminal while high doses have an inhibitory effect on the postsynaptic target (Campbell et al., 2007). Thus, depending on the type of preparation and dosage, kainate might excite or inhibit the presynaptic terminal. The evidence suggests a mechanism of action via kainate-sensitive glutamatergic autoreceptors through a metabotropic action within the presynaptic terminals or by direct action on the presynaptic voltage-gated Ca^{2+} channels (see reviews Lerma, 2003 and Nicoll et al., 2000). In addition, the actions of glutamate at newly hatched *Drosophila* larvae revealed presynaptic actions in enhancing the occurrences of spontaneous events without any alteration in the amplitude of the quantal currents (Zhang et al., 1999). The mechanism suggested for this phenomenon was that presynaptic autoreceptors are present which enhance synaptic transmission. In addition, the pharmacological agonist (1S, 3S)-ACPD for mGluR mimicked the effect of glutamate (Zhang et al., 1999). This helps to establish that autoreceptors at this NMJ are likely in the 3rd instar and that there may be developmental differentiation in presynaptic receptor subtypes to account for the depressing action of kainate in the 3rd instar.

The purpose of this study is to test for these possibilities. We have approached this by accessing the characteristics of single quanta (i.e., peak amplitude) and frequency of spontaneous events with intracellular measures of miniature EPSPs (mEPSPs) from the whole muscle. We also examined further pharmacological profiles of the *Drosophila* NMJ to build knowledge of this model synaptic preparation.

2. Methods

2.1. Electrophysiology and staging of flies

All the electrophysiology and staging of larvae are routine procedures and have been described previously. The dissection technique was previously reported (Ball et al., 2003; Campos-Ortega and Hartenstein, 1985; Li et al., 2002). The physiological saline is HL3 (in mM): 1.0 $CaCl_2$ $2H_2O$, 20 $MgCl_2$, 70 NaCl, 5 KCl, 10$NaHCO_3$, 5 trehalose, 115 sucrose, 5 BES (N,N-bis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid) and adjusted to a pH of 7.2 (Stewart et al., 1994). All experiments were performed at room temperature (20–21 °C). The recording techniques have been previously described for intracellular EPSP measures (Dasari and Cooper, 2004; Sparks et al., 2004; Stewart et al., 1994) and extracellular focal quantal recordings (Cooper et al., 1995a; Harrison and Cooper, 2003). The compound amplitude of the excitatory postsynaptic potentials (EPSP) elicited by Is and Ib motor nerve terminals in segment 3 of muscle m6 were monitored as in Kurdyak et al. (1994) and Ruffner et al. (1999). The identified m6 muscle was used in each preparation. Only preparations with a resting membrane potential of −50 mV or greater were used and the HL3 dissection medium was completely replaced by HL3 media containing the pharmacological compounds. The same time course of pharmacological exposures were used throughout this study.

2.2. Quantal analysis

All quantal analysis of intracellular whole muscle recordings was performed as described previously (Cooper et al., 1995b). The quantal analysis of measuring spontaneous frequency and characteristics of the quantal shape are standard approaches.

2.3. Pharmacology

In an earlier report (Bhatt and Cooper, 2005), actions of NMDA, AMPA, L-glutamate, kainate, quisqualic acid, NBQX, AP5, and DNQX, with regard to synaptic transmission and direct effects on the muscle fibers, were examined. That study showed that kainate reduced evoked EPSPs. The same procedures for exchanging the bathing medium with pharmacological agents were used in the current study. The compound trans-(±)-1-Amino-1,3-cyclopentanedicarboxylic acid monohydrate (t-ACPD) is a known mGluR agonist (Holscher et al., 1997) and was shown to have some effect on newly hatched *Drosophila* larvae (Zhang et al., 1999). We examined the actions of ATPA and SYM 2081, that are known agonists of the kainate receptor in vertebrates, and 1, 3-ACPD on synaptic responses. We also tried an AMPA receptor antagonist GYKI 52466. The ATPA, GYKI 52466, domoic acid and the trans-(±)-1-Amino-1,3-cyclopentanedicarboxylic acid monohydrate (t-ACPD) were obtained from Sigma. SYM 2081 was obtained from TOCRIS.

2.4. Statistical analysis

A non-parametric analysis (Wilcoxon rank sum) or a parametric Student's *t*-test was used for assigning a significance at a $p<0.05$ depending on the appropriate experimental design as explained in the Results section.

2.5. Sequence comparisons

All of the glutamate receptor amino acid sequences used are annotated in the National Center for Biotechnology Information (NCBI) database. There are 36 different genes encoding 36 different kinds of glutamate receptor subunits in *Drosophila melanogaster*. Among them, five subunits consisting of postsynaptic receptors were compared to five different kainate type glutamate receptor subunits in *Homo sapiens*. Both whole protein and extracellular domain sequences of each subunit were aligned to show the similarity and calculate the statistical matches between two species, respectively. The bl2seq in NCBI and Clustal W were used to align two different protein sequences. In Clustal W the default settings were used except ITERATION to alignment and NUMERITER was set to 3.

3. Results

In measuring the evoked EPSPs and spontaneous quantal events a 3rd instar larvae is filleted so that each segment is observed. By stimulating a selected segmental nerve, only one nerve root is recruited which is important since the muscle fibers are electrically coupled between segments. Various identified muscles with the rather simplistic innervation profiles are shown in Fig. 1A. In these studies, we utilized muscle 6 (m6) because of the well characterized innervation as depicted in Fig. 1B (Atwood et al., 1993; Kurdyak et al., 1994; Li et al., 2002). The synaptic properties of the Is and Ib motor nerve terminals have been previously described. We obtained similar measures as earlier reported. We did ensure to observe all three sized EPSP events (Ib, Is and Ib + Is) in order to subsequently monitor the compound Ib + Is EPSP. The resting membrane potential was also monitored and only preparations which did not appear damaged or with a resting membrane potential more negative than −50 mV, in normal physiological saline, were used for assessing the effects of various pharmacological agents.

Application of a saline containing kainate (1 mM) rapidly reduces the EPSP amplitude and the effect is partially reversed by extensively exchanging the bathing media back to normal saline (Fig. 2A). The extant in reduction of the EPSP amplitude is dose-dependent, with an almost 50% reduction at 1 mM (Fig. 2B). All three concentrations produced a significant reduction in the EPSP amplitudes ($n=6$ for each concentration; $p<0.05$ Wilcoxon rank sum non-parametric). There is no significant effect on the resting membrane potential of m6 for any of the three concentrations tested (Fig. 2C). However when considering sham control experiments of exchanged bathing medium with saline and providing the same duration of time in which EPSP measures were made there is a run down in the EPSP amplitude by 13.5% (Fig. 2D). The individual values are shown next to the mean

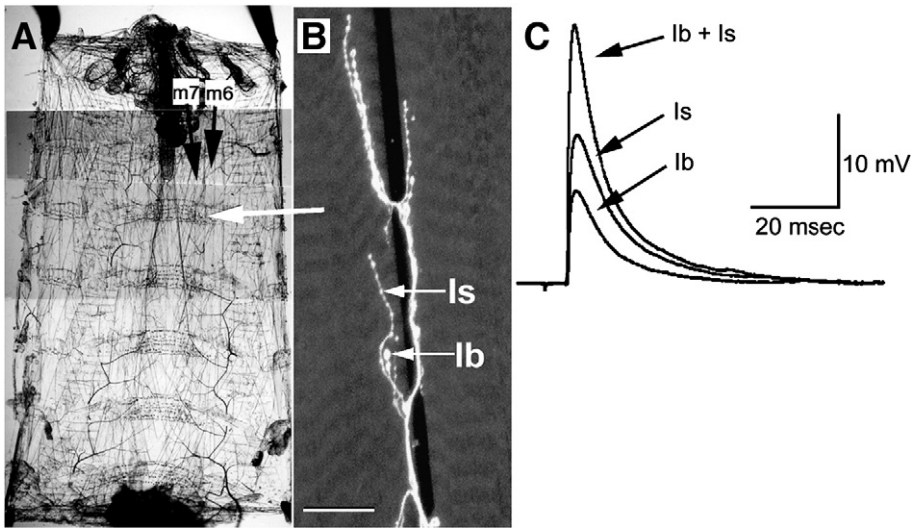

Fig. 1. The dissected 3rd instar *Drosophila* larva preparation. (A) The preparation is pinned at the four corners to keep the preparation taut. The ventral abdominal muscle m6 is used in this study. The segmental nerves are stimulated by placing the nerve into a suction electrode and recruiting the Is and Ib motor neurons. (B) The terminals of Ib and Is on m6 and m7 are readily observed after treatment with fluorescently tagged anti-HRP antibody (Scale: 50 μm). (C) Elicited excitatory postsynaptic potentials (EPSPs) in m6 are measured with a intracellular recording. Representative individual responses from the Ib and Is motor axons as well as the composite Ib and Is response are shown for a early 3rd instar (modified from Bhatt and Cooper, 2005).

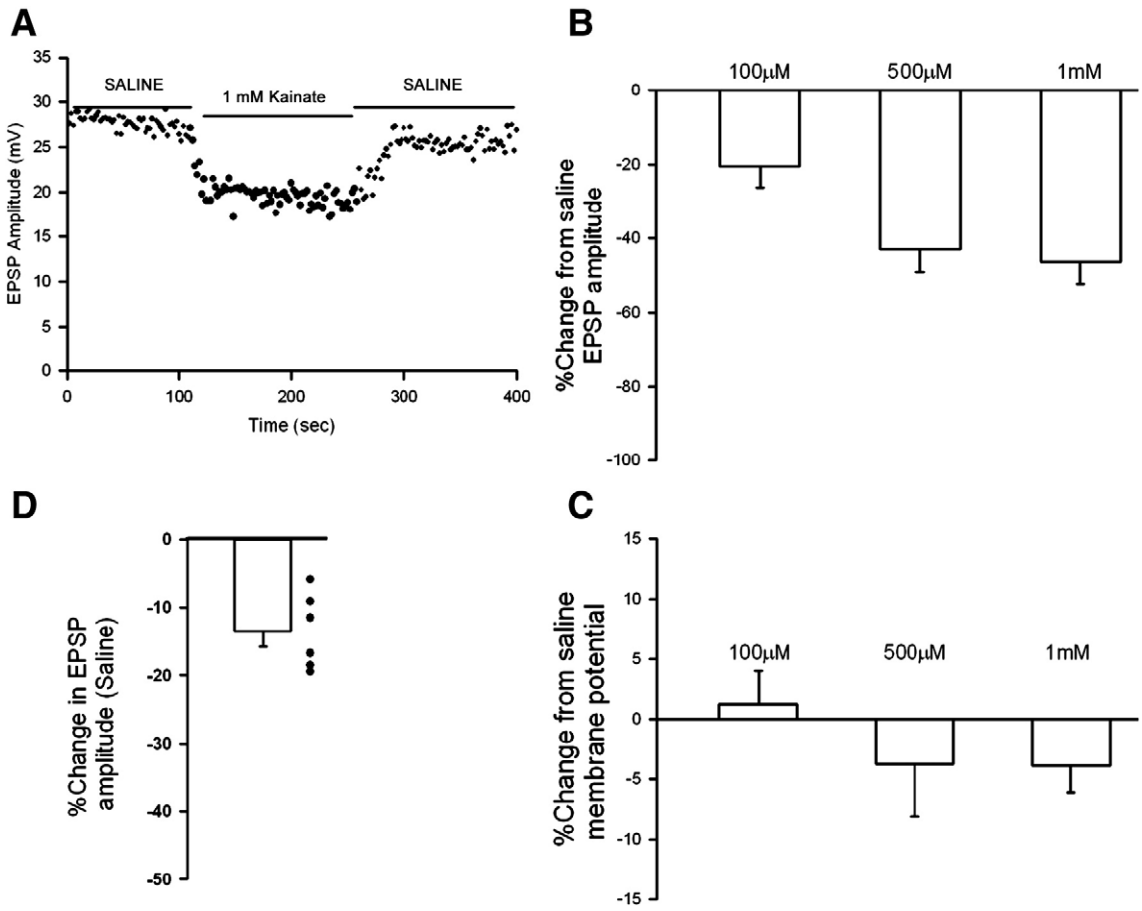

Fig. 2. The effect of kainate on the evoked EPSP amplitudes. (A) Application of 1 mM kainate reduces the EPSP amplitude rapidly and the effect can be partially washed out. (B) The dose–response effect in damping the EPSP amplitude. (C) The dose–response effects on the resting membrane potential for m6. (D) Sham control for exchanging the saline bath and time revealed a 13.5% drop in the EPSP amplitude.

value. The sham procedures produced a significant effect in reducing the EPSP responses ($n=6$; $p<0.05$ Wilcoxon rank sum non-parametric). The mean value was not subtracted from experimental conditions with pharmacological agents in the remaining results, but used to compare the effect to pharmacological agents. Thus, exposure to 500 μM and 1 mM kainate produced a significant difference from sham control whereas 100 μM did not ($p<0.05$ Student's *t*-test).

Similar results were obtained for domoic acid as for kainate. The mEPSP amplitudes and the frequency are reduced (Fig. 3A, B). Also the resting membrane potential is not significantly altered. In examining subtle effects on single quantal events the shape of the quantal events were examined. The amplitude and area (i.e., area under the curve) of the mEPSPs both decreased in a dose-dependent manner for 1 mM and 500 μM (Fig. 3C). With exposure to kainate, the smaller mEPSPs appeared to fade into the background noise while the previously larger mEPSPs events are likely observed as smaller mEPSPs.

In examining the effects of the AMPA and the kainate receptor agonist, domoic acid (10 μm and 1 mM), the postsynaptic receptor sensitivity to spontaneous quantal responses and the frequency of spontaneous events were assessed. It was readily apparent that for kainate, as for domoic acid, a 1 mM rapidly reduced the amplitude of spontaneous mEPSP quantal events (Fig. 4A) as well as the frequency of occurrences (Fig. 3B). Domoic acid at 10 μM or 1 mM had little effect on the resting membrane potential (Fig. 4C). Glutamate at 10 mM produced similar effects in reducing the mEPSP amplitude and frequency but this was compounded by the rapid depolarization that glutamate produced ($p<0.05$; Fig. 4C). The differences in the AMPA and kainate receptor agonist and glutamate is that domoic acid did not significantly depolarize the muscle; however, the mEPSPs were still affected. The combination of domoic acid (1 mM) and glutamate (10 mM) produced a reduced depolarization of the muscle and as expected, a reduced mEPSP amplitude and frequency occurred (Fig. 4B,C). These results suggest domoic acid is an antagonist to the postsynaptic glutamate receptors. The effect on the reduced frequency of the mEPSPs is due to the gradual reduction in the mEPSP amplitude, such that they are not discernable from noise in the baseline and thus are not detected to monitor their frequency.

Since the postsynaptic glutamate receptors on the muscle appeared to bind kainate, we expected the known agonist of kainate receptors 'ATPA' to act similarly, despite knowing that the receptors are not a kainate subtype. ATPA at 1 mM did not reduce the evoked EPSPs (Fig. 5A, B) nor did ATPA have any effect on the resting membrane potential (Fig. 5C). In fact, some preparations increased in the EPSP amplitude while others decreased, thus producing no net significant effect. Since there was no significant effect mEPSPs were not analyzed.

To broaden the survey of pharmacological agents we used SYM 2081, which is a potent and highly selective kainate receptor agonist, with an IC_{50} for inhibition of [^{3}H]-kainate binding of 35 nM and almost 3000- and 200-fold selectivity for kainate receptors over AMPA and NMDA receptors respectively. In addition, we examined a selective allosteric AMPA receptor antagonist GYKI 52466 (Lodge, 2009). But GYKI 52466 at even 100 μM would not dissolve in HL3 saline. This might be due the salt concentration and solubility in HL3 compared to water. So we could not further assess the actions of GYKI 52466 as it would not dissolve in the media. As far as we know, these compounds have not been tried before at the *Drosophila* NMJ. Surprisingly, SYM2081 at 1 mM depolarized the muscle in 6 out of 6 preparations

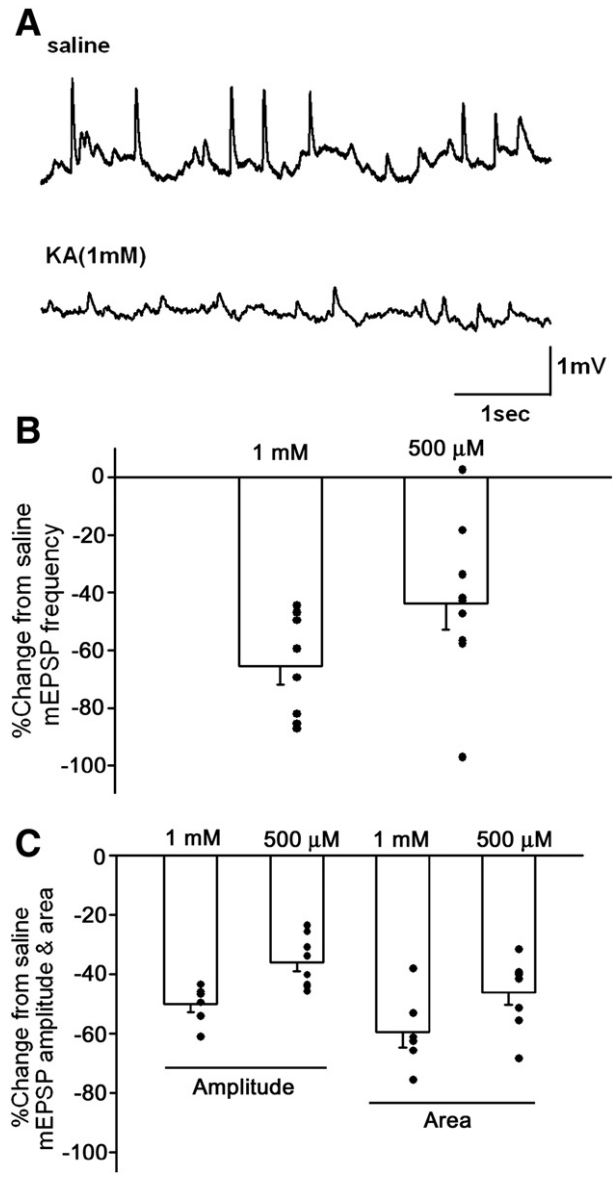

Fig. 3. Effects of kainate (1 and 0.5 mM) on mEPSPs properties. (A) Spontaneous or mEPSPs (miniature EPSPs) are reduced in amplitude upon exposure. (B) The frequency of mEPSPs is reduced by kainate in a dose-dependent manner, as well as the (C) amplitude and area of the mEPSPs.

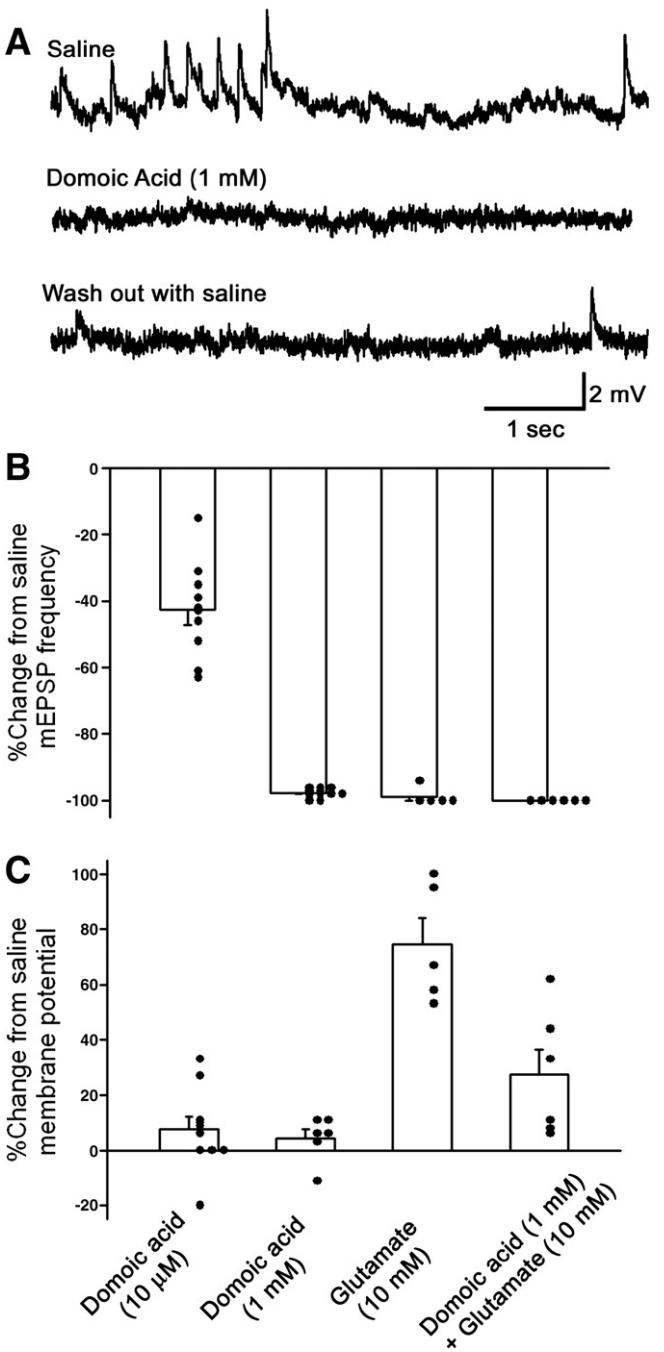

Fig. 4. Effects of domoic acid (agonist for AMPA and kainate receptors) at the NMJ. (A) Spontaneous or mEPSPs (miniature EPSPs) are reduced in amplitude upon exposure to domoic acid. (B) The frequency of mEPSPs is reduced by domoic acid in a dose-dependent manner. Glutamate mimics the effect. (C) Domoic acid results in a slight depolarization of the muscle but not as drastic as glutamate. Domoic acid attenuates the effect of glutamate on membrane depolarization.

(Fig. 6A; $n=6$; $p<0.05$ Wilcoxon rank sum non-parametric). On average there was a 12% increase (less negative) in the resting membrane potential (a 100% change would be considered 0 mV). The SYM 2081 (1 mM) also presented a 40% decrease in the EPSP amplitude which was more of a decrease than expected given and slight depolarization in the muscle (Fig. 6B; $n=6$; $p<0.05$ Wilcoxon rank sum non-parametric). However, SYM 2081 at 0.1 mM had no significant effect on membrane depolarization or on the amplitude of the EPSP.

If there are subtle effects of kainate in depressing presynaptic vesicular docking or altering $[Ca^{2+}]_i$ dynamics, one would expect that facilitation or the short-term depression (i.e., negative facilitation) index might detect such an effect. A short four pulse train of 20 Hz produces the characteristic response known for this preparation, as shown in Fig. 7A. The initial EPSP is normally used as a relative references to the 4th EPSP in the train. It has been previously demonstrated that 1 mM $[Ca^{2+}]_o$ will produce a marked depression in the response train where as 500 µM $[Ca^{2+}]_o$ produces less depression and sometimes a slight facilitation. Thus, both 1 mM and 500 µM $[Ca^{2+}]_o$ were examined with 1 mM and 500 µM kainate to unmask any ceiling effect in the depression by 1 mM $[Ca^{2+}]_o$ exposure. The 1st and 4th EPSPs rapidly attenuate in amplitude upon exposure to 1 mM kainate and 0.5 mM $[Ca^{2+}]_o$ (Fig. 7B). The EPSPs also show a large reduction to kainate when bathed with 1 mM $[Ca^{2+}]_o$ (Fig. 7C). However, no significant differences, by parametric analysis (Student's *t*-test), in the facilitation index are observed among the four conditions for exposure to kainate. This is determined by the difference in the median FI values. The difference is measured from saline to exposure of kainate within each preparation ($n=5$; 500 µM kainate–1 mM $[Ca^{2+}]_o$; 1 mM

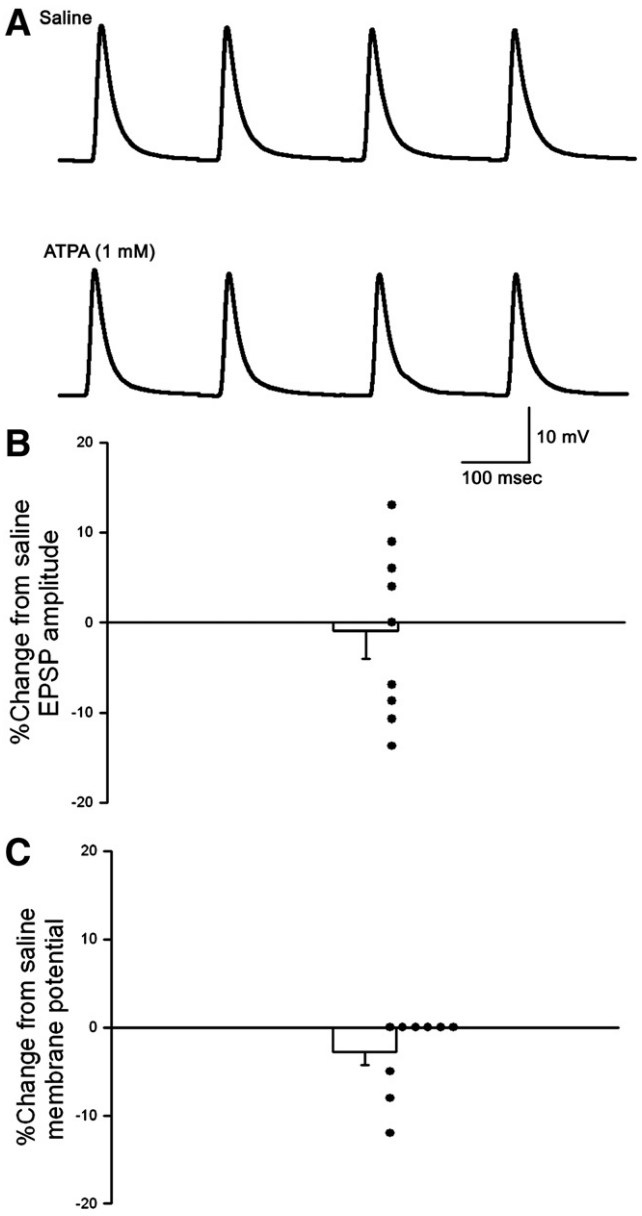

Fig. 5. Effects of ATPA (1 mM; a selective agonist of vertebrate GluR5, kainate receptors) on the amplitude of evoked EPSPs (A) and mEPSPs (B) as well as the resting membrane potential of the muscle (C).

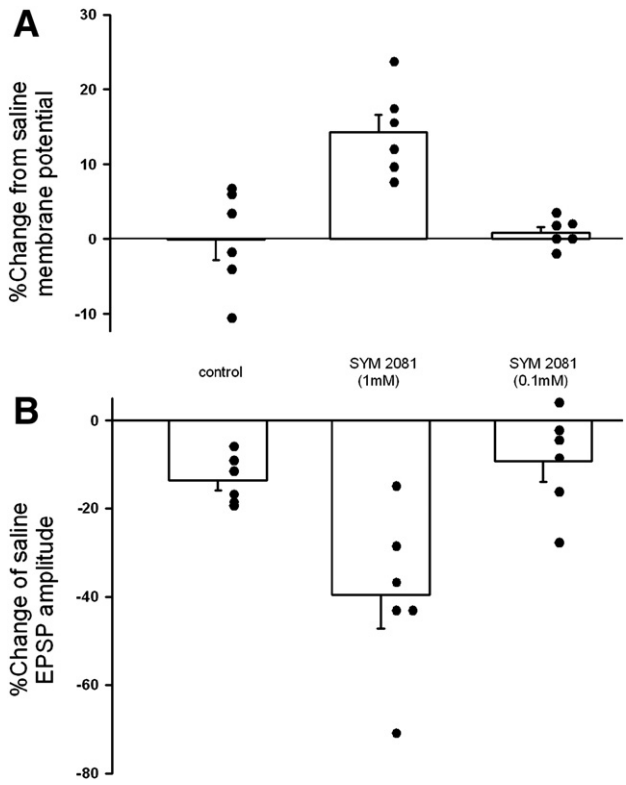

Fig. 6. SYM 2081 at 1 mM depolarizes the muscle (A; $n=6$; $p<0.05$ Wilcoxon rank sum non-parametric). There is mean 12% increase (less negative) in the resting membrane potential. No effect for negative control or for SYM 2081 at 0.1 mM. SYM2081 (1 mM) also presented a 40% decrease in the EPSP amplitude which was more of a decrease than expected given and slight depolarization in the muscle (B; $n=6$; $p<0.05$ Wilcoxon rank sum non-parametric). At 0.1 mM SYM2081 there is no difference from the negative control on the amplitude of the EPSPs.

kainate–1 mM $[Ca^{2+}]_o$; 500 μM kainate–500 μM $[Ca^{2+}]_o$; 1 mM kainate–500 μM $[Ca^{2+}]_o$; Fig. 7D). If the effect to exposure of kainate (1 mM) with 1 mM $[Ca^{2+}]_o$ is analyzed by non-parametric statistics, then five out of five preparations showed an increase in the mean FI ($p<0.05$, Wilcoxon Rank Sum). However, two preparations showed only small increases of 10 and 12%. Thus, we do not feel confident in stating that there is a significant increase in the mean FI with kainate by non-parametric analysis. On the other hand, box-whisker plots of the FI values before and after exposure to kainate, for each preparation, in the various paradigms revealed a trend again related to the same data set of kainate (1 mM) with 1 mM $[Ca^{2+}]_o$ (Fig. 8A). The box-whisker plots represent the median, 25th, 75th, and range. One can observe that the variability in the distributions is larger for the kainate (1 mM) with 1 mM $[Ca^{2+}]_o$ as compared to the same preparations before the exposure. In the other paradigms the variability is about the same for saline and kainate exposure. To further examine this variability in FI, the standard deviation for each preparation before and after exposure to kainate was examined. Only the kainate (1 mM) with 1 mM $[Ca^{2+}]_o$ showed a consistent trend (five out of five; $p<0.05$, Wilcoxon Rank Sum Test; also by Student's *t*-test $p<0.05$) with an increase in the variability while exposed to kainate as compared to the saline baseline. The change in the individual preparations for this paradigm are shown in Fig. 8B.

To further investigate possible presynaptic, metabotropic glutamate receptor action on the motor nerve terminals, the common metabotropic glutamate receptor agonist trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) was used. Potentially, an increase or a decrease in the evoked EPSP would be possible at this insect NMJ since at the crayfish NMJ (also an arthropod species) an effect was reported (Schramm and Dudel, 1997). However, no significant alterations on evoked EPSPs amplitudes (Fig. 9A, B) or on the resting membrane potential of the muscle occurred during exposure to t-ACPD (500 μM). In comparison to the sham saline control (Fig. 2D), the distributions in EPSP amplitudes over lap; thus, the slight reduction in EPSP amplitudes with t-ACPD are not significant.

4. Discussion

In this study we showed, in a concentration-dependent manner, that kainate rapidly reduced the amplitude of evoked EPSPs as well as the spontaneous quantal events. The lack of any response to t-ACPD and ATPA as well as the inability of these compounds to mimic kainate's action suggest that there is not a presynaptic mechanism of kainate-like or metabotropic autoreceptors on these motor nerve terminals. However, the kainate receptor agonist, domoic acid, did follow the actions of kainate in reducing the evoked EPSPs. We suggest this action is due to domoic acid and kainate acting as an antagonist on the postsynaptic quisqualate receptors present on

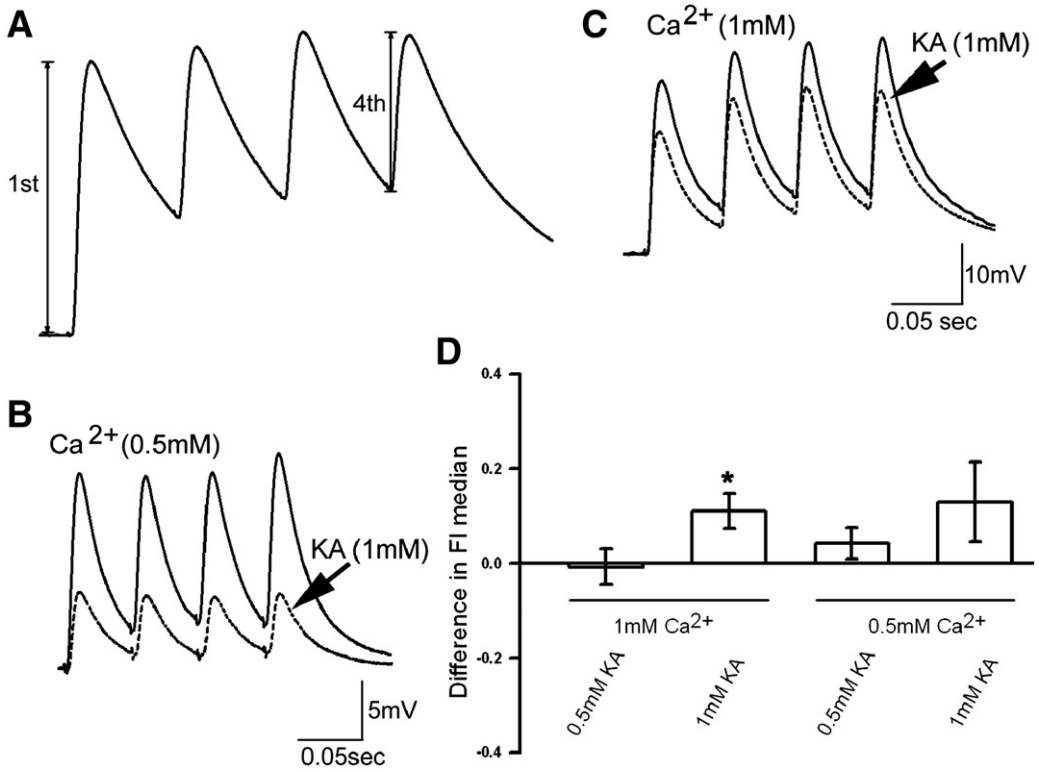

Fig. 7. Effects of kainate on short-term depression (i.e., negative facilitation) with varied $[Ca^{2+}]_o$. (A) A four pulse stimulus train delivered at 20 Hz results in an initial large EPSP followed by depressed responses. The procedure for measuring EPSP amplitudes is indicated. Facilitation index (FI) is then calculated from these measures. (B) A typical response for the effect of kainate (KA; 1 mM) on the pulse train in the presence of 0.5 mM $[Ca^{2+}]_o$. (C) A typical response for the effect of KA (1 mM) on the pulse train in the presence of 1 mM $[Ca^{2+}]_o$. (D) The difference in facilitation index (FI) is shown for both 1 mM and 0.5 mM KA with either 1 mM or 0.5 mM $[Ca^{2+}]_o$. The difference in the median values are obtained from the difference in FI saline to exposure of KA within each preparation. ($n=5$ for each paradigm; * $p<0.05$ Wilcoxon Rank Sum Test for the effect of KA as compared to saline).

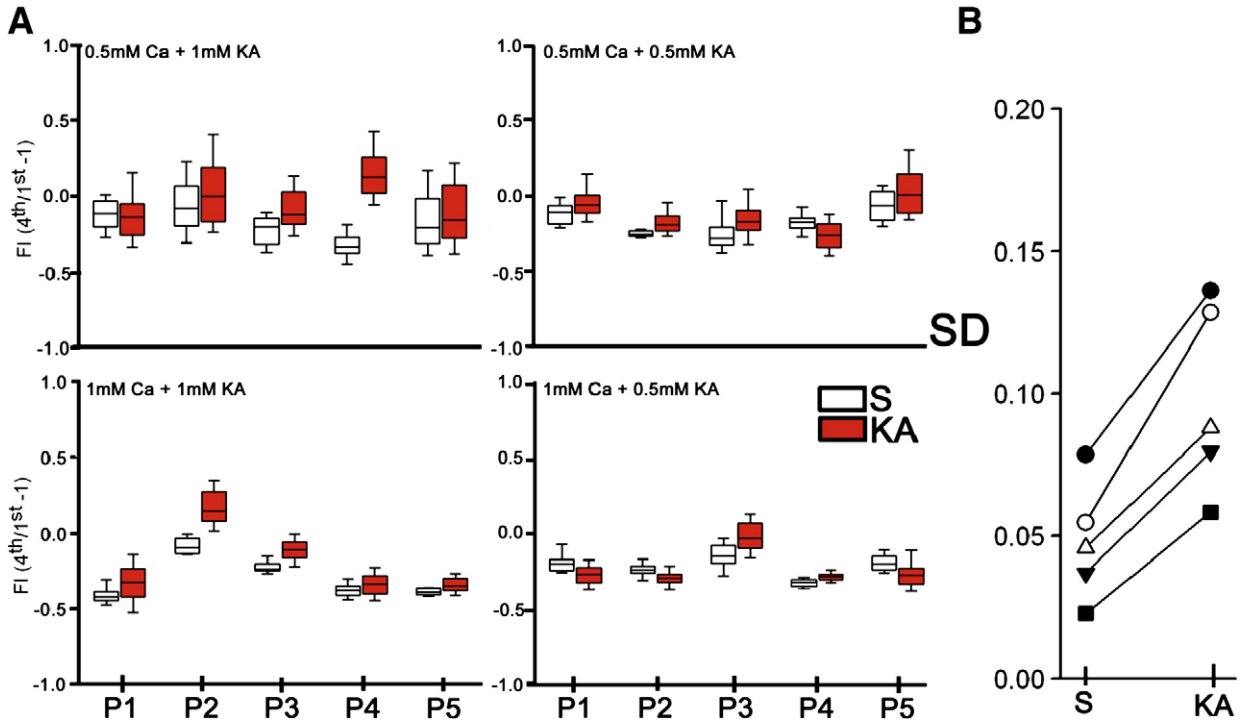

Fig. 8. (A) Effects of kainate (KA) and calcium on FI. Box and whisker plots for FI in various conditions are shown as before and after exposure to kainate, for each of the five preparations in the four experimental conditions. The plots show the median,10th, 25th, 75th, and 90th percentiles. (B) The standard deviation (SD) for each preparation before and after exposure to kainate (1 mM) with 1 mM $[Ca^{2+}]_o$ presented a consistent trend in increasing in the variability among the data sets while exposed to kainate (KA) as compared the saline (S) baseline ($p<0.05$, Wilcoxon Rank Sum Test; also $p<0.5$ Student's t-test).

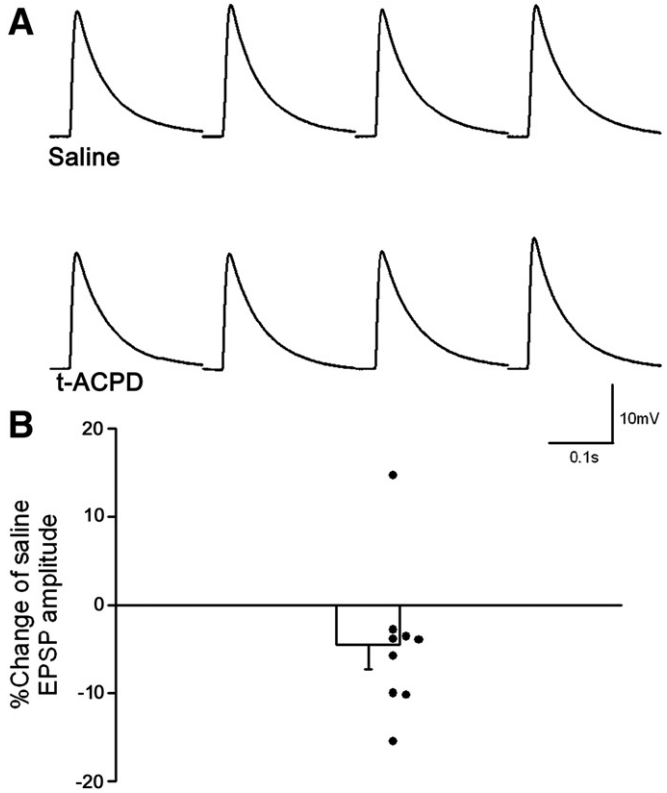

Fig. 9. Action of t-ACPD on evoked EPSPs and resting membrane potential. (A) Evoked EPSPs in saline and during exposures to t-ACPD (50 μM). (B) The mean percent change in the amplitude of the evoked EPSPs with exposure to t-ACPD. (C) The mean percent change in the resting membrane potential with exposure to t-ACPD.

muscle and that no presynaptic contribution can be proposed for the rapid reduced amplitude of evoked EPSP and quantal responses. The agonist action of SYM 2081 at high concentration suggests that this compound may have some action on quisqualate receptors, perhaps even in other invertebrates. However, SYM 2081 does behave like kainate in this preparation. The reduced frequency in the spontaneous quantal responses in the presence of kainate or domoic acid appears to be due to the responses decreasing in size so they are not detectable from the noise or that the postsynaptic receptors are fully blocked.

The responses observed at the crayfish NMJ by Schramm and Dudel (1997), were anticipated to be analogous to the *Drosophila* larval NMJ that we used in our study since they are similar in general function and structure (Atwood and Cooper, 1995, 1996), but there must be differences in regards to presynaptic autoreceptors. Schramm and Dudel (1997) reported that some preparations showed that t-ACPD and glutamate itself produced varied responses of mean quantal content. Some preparations they reported showed an enhancement while others a decrease in evoked postsynaptic events. Schramm and Dudel (1997) concluded that there may be differential regulation in the type and density of the metabotropic presynaptic autoreceptors to account for variation among preparations and effects to t-ACPD and glutamate. There are methodological differences in stimulating the nerve terminals. In our studies, we elicited action potentials within the nerve to result in Ca^{2+} influx and vesicular fusion. However, Schramm and Dudel (1997) used depolarization pulses delivered over the terminal, through the focal electrode, to directly open voltage-gated channels. Seal stability with the focal electrode over the NMJ, longer than normal action potential depolarization pulses and tweaking the current pulses to a fine degree in order to balance a percentage of activating voltage dependent ion channels, would appear to induce potentially more experimental variability among preparations than inducing a standard action potential within the nerve for evoking synaptic transmission. The large field effect of a current pulse, to induce presynaptic depolarization, through a macropatch electrode could have an effect on the postsynaptic glutamate receptors such that the receptor interaction with pharmacological agents of even glutamate itself might be altered. To resolve these issues in the crayfish would require selectively stimulating the motor nerve and repeating the study.

As for domoic acid, the results suggest kainate is an antagonist to the postsynaptic glutamate receptors in a dose-dependent manner, but not ATPA. In addition, since ATPA did not produce any significant alteration in the evoked EPSP amplitude, there does not appear to be any pre- or post-synaptic action of ATPA. Quisqualate has been suggested to activate ionotropic as well as metabotropic glutamate receptors just as glutamate does itself, which suggests quisqualate is a non-selective glutamate receptor agonist (Schoepp et al., 1990). However, AMPA, which is significantly similar to quisqualate in structure and is a weak kainate receptor agonist, cannot activate nor even bind to homomeric GluR6 kainate receptor (Egebjerg et al., 1991). Such differences may be induced by steric hindrance near the ligand binding domain. In fact, AMPA did not change the EPSP amplitude in our studies, but kainate resulted in reduced amplitude by acting as an antagonist. Therefore, there seems to be other factors besides steric hindrance. It would seem likely that a ligand binding site of kainate at the *Drosophila* NMJ would be highly conserved to the vertebrate kainate receptor, since kainate can block evoked responses; however, the similarity of extracellular domains is not very high. One would not have expected from the results with AMPA and domoic acid that the potent and highly selective kainate receptor agonist SYM 2081 to behave as glutamate. However, it does mimic glutamate but with less affinity. Recent studies suggested that the properties of ionotropic glutamate receptor subunits can be changed by mutating the amino acid at the second transmembrane segment which forms the lining of the pore region (Bochet et al., 1991; Curutchet et al., 1992; Verdoorn et al., 1991). For the functional muscle-expressed glutamate receptor GluR-IIC, GluR-IID, GluR-IIE and either GluR-IIA or GluR-IIB are essential for function. Therefore, only based on the ligand binding domain, tetrameric structure was suggested. The correct subunit stoichiometry is not known. The receptor can be pentameric or greater such as (IIA)(IIB)(IIC)(IID)(IIE) or (IIA)(IIA)(IIC)(IID)(IIE) to be functional. Also, glutamate receptor complexes which contain either GluR-IIA or GluR-IIB have opposite physiological properties by showing slow and fast desensitization, respectively (DiAntonio et al., 1999). Thus, as constituted receptor subunits are put together it is likely that the receptor is functionally changed. There could also be mixed subunit combinations along the nerve terminal among the many synaptic sites, since the terminal is developmental different in age (Atwood and Cooper, 1995; He et al., 1999; Li et al., 2002) and function (Bogdanik et al., 2004; Guerrero et al., 2005; Marrus et al., 2004; Liebl and Featherstone, 2005) along its length. Developmental alteration in kainate receptors is known in vertebrates (Lee et al., 2001). Thus, variability in whole muscle responses to pharmacological agents might be masked, but if older and younger regions of the terminals were monitored with focal macropatch electrodes larger differences in the pharmacological profiles and sensitivities might be observed.

It was proposed that the five subunits forming the postsynaptic receptors in *Drosophila* NMJ are somewhat similar in homology to kainate receptors in vertebrates and thus were termed kainate-like (Betz et al., 1993; Guerrero et al., 2005; Marrus et al., 2004; Qin et al., 2005; Völkner et al., 2000). In comparing the postsynaptic receptor molecular sequence of *Drosophila* NMJ with a kainate receptor in *Homo sapiens*, which contains the highest homology in the vertebrates, each subunit shows a moderate similarity between the two species. Extracellular domains of the receptors are not significantly higher in similarity compared to total sequence (Fig. 10). In a

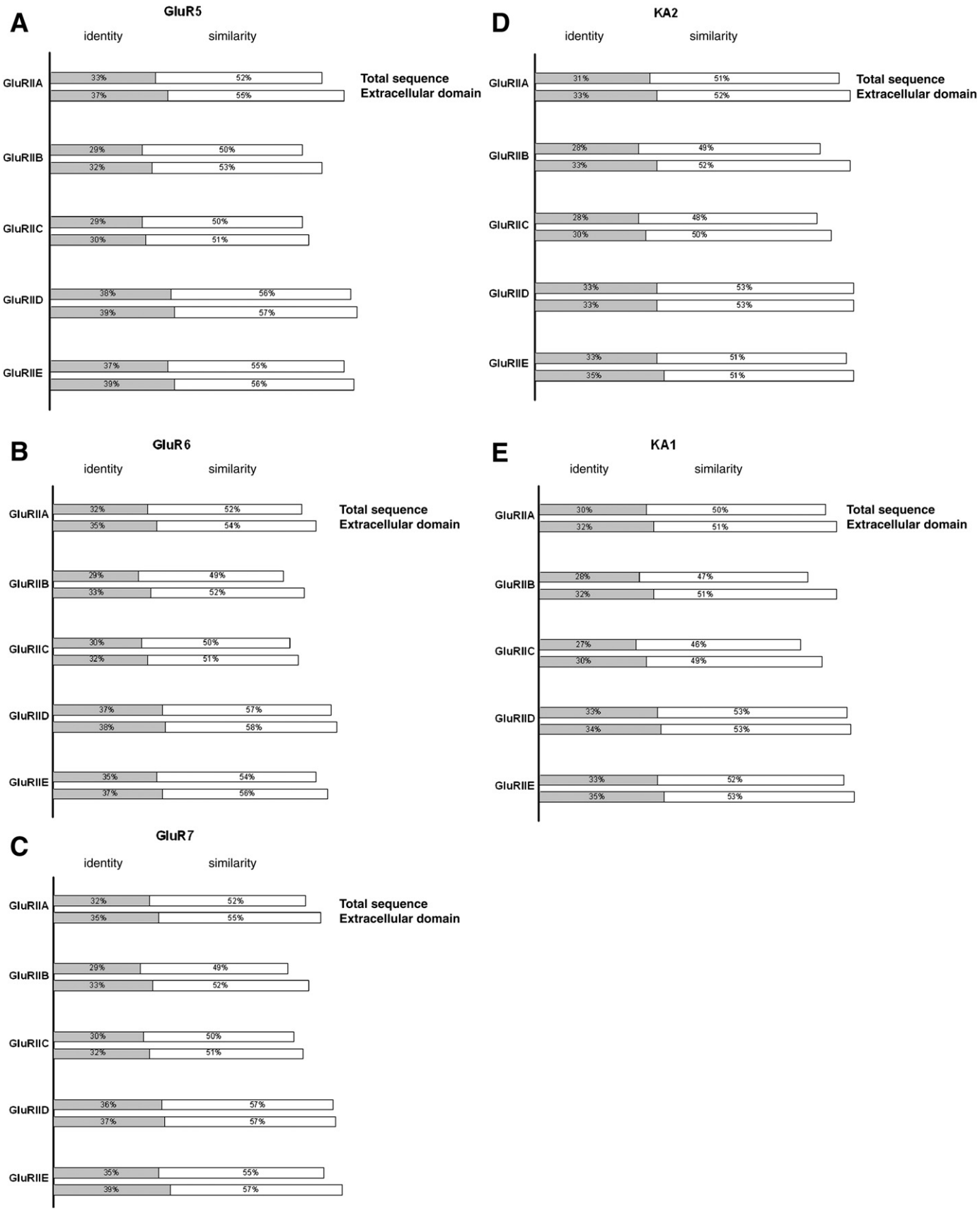

Fig. 10. Sequence comparison of five postsynaptic receptor subunits identified from *Drosophila* muscle mRNA to the five known human kainate receptor subunits. Each two sequences were compared by using a blastP alignment. The top bar is a comparison for the total protein sequence. The bottom bar is a comparison of the putative extracellular domains among the proteins. The gray area (box) is the percentage of identical sequence and the white area (clear box) is the percent in similarity based on the blastP (NCBI) algorithm. The five human receptor subunits used are: (A) GluR5; (B) GluR6; (C) GluR7; (D) KA1; and (E) KA2.

phylogeny tree with 36 glutamate receptor protein sequences in *Drosophila melanogaster* and the human's NMDA, kainate and AMPA receptors, the five subunits of postsynaptic receptors in *Drosophila* NMJ appear to be in a different subgroup to even the closest of the vertebrate kainate/AMPA type receptors (Fig. 11). We do not really understand why these receptors were considered to be 'kainate-like' in previous comparative studies, as they do not fit the pharmacological profile or the molecular sequence.

There are multiple examples of pre- and/or post-synaptic action of kainate in enhancing or suppressing synaptic transmission in vertebrate neuronal circuits (Grabauskas et al., 2007; Hegarty et al., 2007; Kamiya and Ozawa, 2000; Pinheiro and Mulle, 2008; Rozas et al., 2003; Schoepp, 2002); and it has been reported earlier that kainate blocked synaptic transmission at the *Drosophila* NMJ (Chang and Kidokoro, 1996; McLarnon and Quastel, 1988). However, the earlier studies with *Drosophila* did use Schneider's *Drosophila* medium which is known to have a depressing action on synaptic transmission itself (Ball et al., 2003; Stewart et al., 1994). The reduction in EPSPs as shown for the negative control illustrates the need for effects of saline to be compared to the experimental manipulations. The small rundown in the EPSP amplitudes is less of a problem with HL3 as compared to Schneider's *Drosophila* medium (Ball et al., 2003; Stewart et al., 1994). It is likely that HL3 is not an optimally suited saline for muscle maintenance and that the nerve membrane becomes leaky as we did not observe depolarization in the muscle but a reduced evoked response. Thus, there could be reduced transmitter release or reduced sensitivity postsynaptically over time. Since the distribution in amplitude of spontaneous events dose not shift it is likely the saline effect is presynaptic. The time dependent effect in the negative control was compared to the experimental responses for examining the realistic effects of pharmacological agents in these studies but one rarely notes negative controls in other studies with this preparation. Mechanistically we do not know how kainate is blocking transmission, but it appears likely that kainate acts as an antagonist in this preparation. Possibly kainate could act on the receptor to desensitize it to glutamate or in addition act as a non-competitive antagonist. Quisqualate receptors, as well as other glutamate receptors (Sun et al., 2002), are known to be prone to desensitization at the crayfish NMJ (Tour et al., 2000) as well as in other preparations (Sun et al., 2002).

The results with kainate and the two concentrations of bathing $[Ca^{2+}]_o$ is interesting since only the one condition (1 mM kainate and 1 mM $[Ca^{2+}]_o$) produced a significant trend (Fig. 8). As stated earlier the increase in median values is small in some preparations, so we are cautious in bearing too much emphasis on this result; however, the increase in variability when exposed to kainate is of interest. If kainate

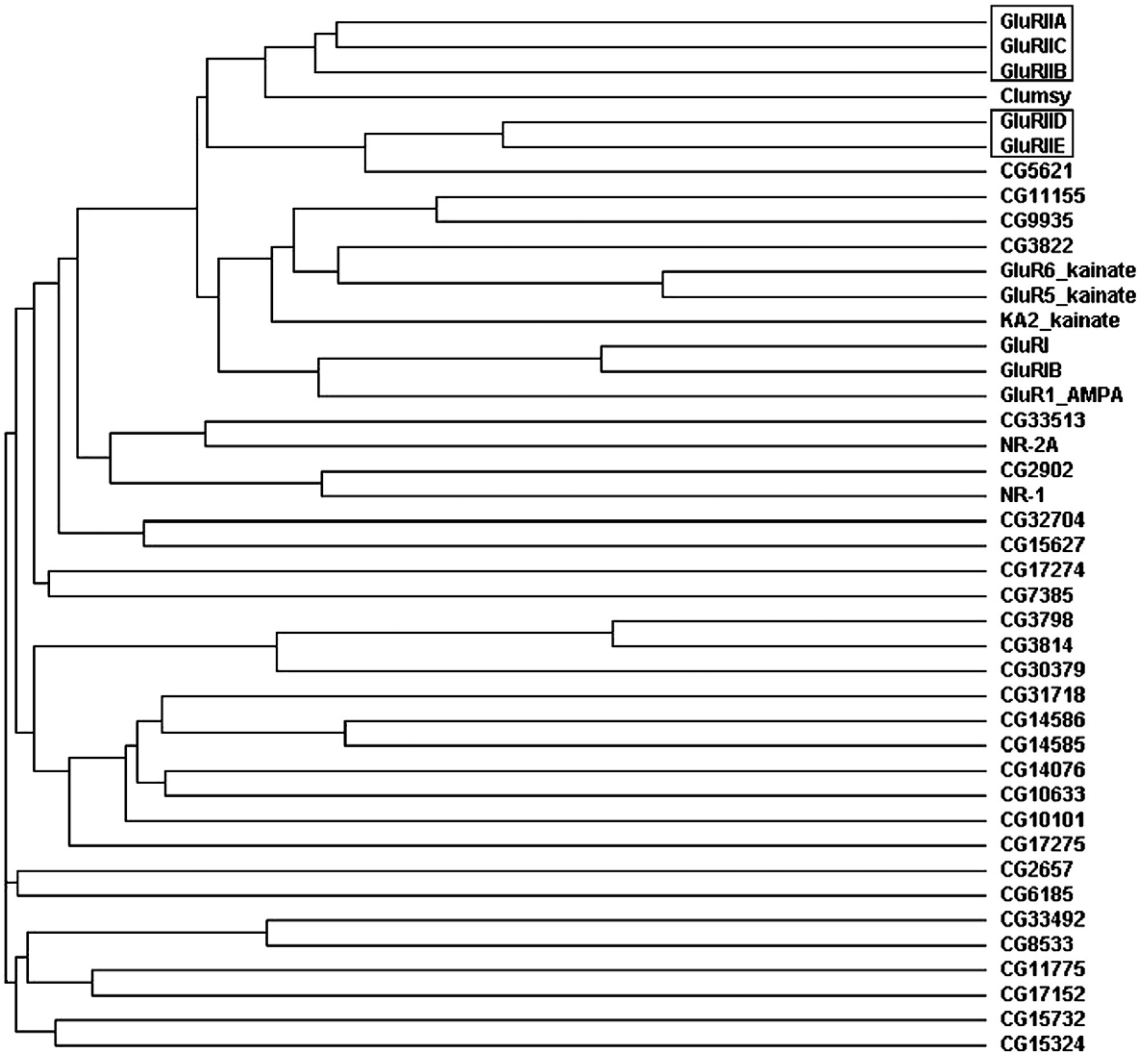

Fig. 11. Phylogeny tree comparing all known putative 36 ionotropic glutamate receptor subunits in the *Drosophila* genome with human glutamate receptors (kainate receptor subunits GluR5, GluR6 and KA2; AMPA receptor subunit GluR1; NMDA receptor subunits NR-1 and NR-2). The comparison was performed by ClustalW2 (EMBL-EBI). The five subunits of postsynaptic receptors in *Drosophila* larval muscle are shown in the boxes.

is acting as a competitive antagonist then it is feasible to speculate with each pulse in the stimulus train the glutamate is resulting in a greater displacement of the kainate and possibly resulting in more competitive-competition. As the glutamate is being taken back up in the nerve terminal by the glutamate transporters, kainate's action can dominate producing very variable responses on the amplitudes of the EPSPs throughout the four pulse train. Considering when saline is exchanged for saline containing kainate, the 1st EPSP within the train is dampened significantly by the antagonistic action and during the evoked release of glutamate then the kainate is displaced would possible explain the slight, but consistent, increase in the median FI values as well for this experimental paradigm. This also follows for the concept that when the $[Ca^{2+}]_o$ is reduced to 0.5 mM, the evoked amount of glutamate is not as pronounced so less displacement of the kainate would occur, which might explain why there is not as much change on FI or on the median as well as the variability in the FI (Fig. 8). We are not aware of reports addressing pharmacological examination of competitive antagonists during pulse trains to account for fluctuation in facilitation measures to compare our findings. More studies are warranted to examine this tentative working model in competitive antagonism during synaptic facilitation/depression to explain the mechanistic details behind these findings.

Since we have not been able to detect any presynaptic auto-receptor function at these NMJs, it would be interesting to know if these motor neurons produce mRNA for any glutamate receptor subtype. There has yet to be investigation of possible presynaptic actions at other NMJs in the larvae or adult of *Drosophila*. The subset of junctions that we examined may not be indicative of all the *Drosophila* NMJs, so further investigations are warranted.

Acknowledgments

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