SHORT COMMUNICATION

The pharmacological and physiological profile of glutamate receptors at the *Drosophila* larval neuromuscular junction

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Abstract. *Drosophila* larval muscles are commonly used for developmental assessment in regard to various mutations of synaptically relevant molecules. In addition, the molecular sequence of the glutamate receptors on the muscle fibre have been described; however, the pharmacological profiles to known agonists and antagonists have yet to be reported. Here, the responses of *N*-methyl-D-aspartic acid, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), L-glutamate, kainate, quisqualic acid, NBQX, AP5 and DNQX are characterized with regard to synaptic transmission and direct effects on the muscle fibres. The muscle fibres depolarize to application of glutamate or quisqualate and the excitatory post-synaptic potential (EPSP) amplitudes are diminished. Kainate does not alter the muscle membrane potential but does reduce the EPSP amplitude. The known antagonists NBQX, AP5 and DNQX have no substantial effect on synaptic transmission at 1 mM, nor do they block the response of quisqualate. Kainate may be acting as a postsynaptic antagonist or via autoreceptors presynaptically to reduce evoked transmission.

Key words. Behaviour, development, excitatory amino acid, insect, locomotion

Introduction

The neurotransmitter at the neuromuscular junction (NMJ) associated with skeletal muscles in the larval *Drosophila* is assumed to be glutamate because receptors sensitive to exogenous L-glutamate are present on the muscle (Jan & Jan, 1976). The muscle is most sensitive to glutamate compared with other potential candidate transmitters, such as aspartate or actelycholine. In vertebrates, glutamate receptors within the central nervous system are defined in relation to ion flux characteristics and *N*-methyl-D-aspartic acid (NMDA) sensitivity as NMDA or non-NMDA subtypes (i.e. quisqualate, AMPA and kainate). Some glutamate receptors in vertebrates are metabotropic and mediate their action through the activation of second messenger cascades, such as in quisqualate-activated receptors.

However, at the insect and crayfish NMJ, the glutamate receptors are most prominently activated by quisqualate and are ionotropic (Shinozaki & Shibuya, 1974; Anderson et al., 1976; Patlak et al., 1979; Gration et al., 1981; Shinozaki & Ishida, 1981; Cull-Candy & Parker, 1983; Bhatt et al., 2004). Ionotropic receptors are ligand-gated ion channels. Thus, some similarity in function of insect glutamate receptors at the NMJ is likely compared with vertebrate ionotropic and metabotropic glutamate receptors found in the central nervous system.

According to previous studies, the glutamate receptors at the larval *Drosophila* NMJ act similalrly to the glutamate receptors at the crayfish NMJ in their rapid onset of opening and current flow. At the crayfish NMJ, the glutamate receptors are of the quisqualate-type with rapid inward sodium conductance along with some inward calcium current, as well as some outward potassium flux (Shinozaki & Ishida, 1981; Dudel et al., 1992).

Surprisingly, only recently have the glutamate receptors at the *Drosophila* NMJ been investigated with a wide range of agonists and antagonists to classify their pharmacological profile (Bhatt et al., 2004). Previous studies demonstrate that quisqualate activated glutamate receptors on

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Drosophila muscle with similar channel characteristics and that α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA, an agonist of quisqualate-type receptors) has insignificant effects (Chang & Kidokoro, 1996). Chang & Kidokoro (1996) also demonstrated that kainate (an agonist of glutamate receptors) does not activate embryonic *Drosophila* muscle glutamate channels. By examining the *Drosophila* genome, it is known that there are approximately 30 glutamate receptor genes (Littleton & Ganetzky, 2000). Two types are expressed in muscle, DGluR-IIA and DGluR-IIB, and only a limited degree of investigation of the pharmacology of the receptor types has been conducted. The presence of L-glutamate and L-aspartate leads to the depolarization of *Xenopus* oocytes through expressed DGluR-IIA receptors (Schuster *et al.*, 1991). The same receptors show little sensitivity to quisqualate, AMPA and kainate, in contrast to the glutamate receptors at crayfish NMJs. It is also known that receptor types expressed in frog oocytes may not reveal the true physiological and pharmacological profiles of the endogenous receptors within the native animal. Therefore, the pharmacology of the glutamate receptors can be examined at intact NMJs of the larval *Drosophila*. Kinetic analysis of glutamate receptors in cultured embryonic *Drosophila* myotubes with the patch clamp method reveals that the channels which open briefly are singly liganded openings and bursts that are long in duration are doubly-liganded openings (Chang & Kidokoro, 1996). Further detailed studies on the ion permeability of the *Drosophila* muscle glutamate receptor reveal that the channel is a one-ion pore with three energy barriers and two internal sites, as based on the Eyring's reaction rate theory model (Chang *et al.*, 1994). In addition, activation of a metabotropic glutamate receptor (mGluR) at the *Drosophila* NMJ may be responsible for potentiating synaptic transmission via a process involving cAMP (Zhang *et al.*, 1999).

The findings of the present study are significant because the receptor types at the *Drosophila* NMJ are pharmacologically and physiologically classified. In this true model organism with a known genome, mutational studies are being widely employed to assay developmental and synaptic mechanisms.

Materials and methods

Insects

The wild-type laboratory strain of *Drosophila melanogaster*, Canton S, was used. All animals were maintained in vials partially filled with a cornmeal–agar–dextrose–yeast medium. Larvae at the beginning of the 'wandering' phase of the third instar were used in these experiments. Staging of fly larvae and the dissection technique have been reported previously (Campos-Ortega & Hartenstein, 1985; Li *et al.*, 2002; Ball *et al.*, 2003).

The physiological saline HL3 was used comprising (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-aminoethanesulphonic acid) and adjusted to a pH of 7.2 (Stewart *et al.*, 1994). All experiments were performed at room temperature (20–25 °C).

Neuromuscular physiology

The recording arrangement was the same as previously described (Stewart *et al.*, 1994; Dasari & Cooper, 2004; Sparks *et al.*, 2004). Intracellular recordings in muscles were made with 30–60 MΩ resistance, 3M KCl-filled microelectrodes. The compound amplitude of the excitatory postsynaptic potentials (EPSP) elicited by Is and Ib motor nerve terminals in segment 3 of muscle m6 was monitored. This identified m6 muscle was used in each preparation. Electrical signals were recorded on-line to a PowerMac 9500 via a MacLab/4 s interface and calibrated with the MacLab Scope software (3.5.4 version, AD Instruments, Australia). Only preparations with a resting membrane potential of −50 mV or greater were used. In addition, if the recording was lost during the experiment, the preparation was not used for analysis.

Pharmacology

The HL3 dissection medium was completely replaced by the media containing the pharmacological compounds. The media was rapidly exchanged within 30 s. The agonist NMDA, AMPA agonist, L-glutamate (agonist), kainate (agonist), quisqualic acid (agonist), NBQX (competitive AMPA/kainate receptor antagonist), AP5 (NMDA receptor antagonist), and DNQX (competitive quisqualate receptor antagonist) were obtained from TOCRIS (Ellisville, Missouri; purity >98%). After examining the various pharmacological agents, the medium was changed back to HL3 to examine recovery of synaptic functions.

Analysis

A percentage change is used to show relative differences because slight variations occur in the initial compound EPSP amplitude and the resting membrane potential among preparations. The percentage change was determined by the absolute difference in the initial and experimental condition, which was divided by the initial value. The result was multiplied by 100. A nonparametric analysis (Wilcoxon rank sum) or a parametric Student's *t*-test was used for assigning significance at $P < 0.05$.

Results and discussion

In dissected third-instar larvae, each segmental nerve root and ventral body wall musculature is readily observed (Fig. 1A). Muscle 6 (m6) in segment 3 was utilized because of the well-characterized innervations and synaptic

Fig. 1. Schematic diagram of the dissected 3rd-instar *Drosophila* larva (A). The preparation is opened and pinned at four corners to allow the preparation to remain extended and tight. The prominent m6 (segment 3) was used for experimentation, where the third segmental nerve is cut away from the central nervous system, and pulled into a stimulatory suction electrode. (B) The motor nerve terminals Ib and Is on m6 and m7 imaged with a confocal microscope from a preparation stained with a fluorescent antihorseradish peroxidase (HRP-FITC) primary antibody (ICN Pharmaceuticals, Costa Mesa, California) (Scale bar = 50 μm). (C) Intracellular electrophysiological recordings from m6 during the stimulation of Ib and Is, which can be stimulated singularly, or together, resulting in a compound 'Ib + Is' excitatory postsynaptic potential.
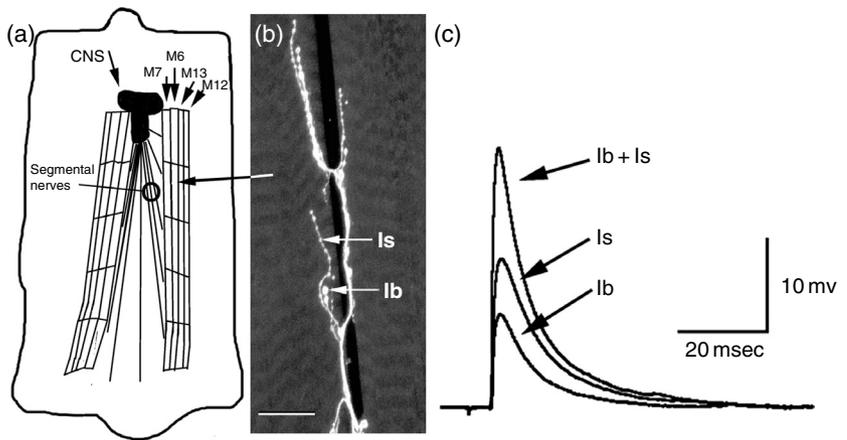
properties of the Is and Ib motor nerve terminals (Fig. 1B) (Atwood *et al.*, 1993; Kurdyak *et al.*, 1994; Li *et al.*, 2002). The third segmental nerve root was stimulated to drive both the Ib and Is motor neurones to allow monitoring of the compound EPSP response (Fig. 1C).

The common glutamate receptor agonists were first assayed at a 1 mM concentration to monitor alterations in the EPSP amplitude (Fig. 2A) and the muscle membrane potential (Fig. 2B). It is apparent that quisqualate and glutamate share a similar response, causing an almost 100% decrease in the EPSP amplitude ($P < 0.05$, nonparametric). Kainate also produces a substantial decrease in the EPSP amplitude (46%; $P < 0.05$, nonparametric). The response to NMDA and AMPA results in only a 5–10% decrease in EPSP amplitude ($P < 0.05$, nonparametric). The antagonist to quisqualate receptor, DNQX (1 mM), and the competitive AMPA/kainate receptor antagonist, NBQX (1 mM), demonstrate a 10–18% ability to reduce the EPSPs ($P < 0.05$, nonparametric). There is no significant effect of AP5 (1 mM) on the EPSP amplitude. The reduction in the EPSP amplitudes arises in part because of the depolarization of the muscle membrane potential. The application of AMPA shows a slight hyperpolarization of the membrane potential in each of the five preparations ($P < 0.05$, nonparametric). NMDA produces a small but consistent depolarization in the muscle of approximately 8% on average ($P < 0.05$, nonparametric). However, quisqualate almost completely depolarizes the muscle to 0 mV (90%; $P < 0.05$, nonparametric) whereas glutamate depolarizes the fibres to approximately 75% of their initial value ($P < 0.05$, nonparametric). Interestingly, kainate does not depolarize the muscle fibres, suggesting no agonist action on the muscle fibres despite decreasing EPSP amplitudes. The antagonists AP5, DNQX and NBQX show no consistent differences in altering the membrane potential.

Because quisqualate and kainate have substantial effects in reducing the EPSP amplitudes, dose–responses at 100 μM, 500 μM and 1 mM are examined. The rapid effect

of the quisqualate at each concentration is shown in Figure 3(A). The rate of the effect is similar for kainate.
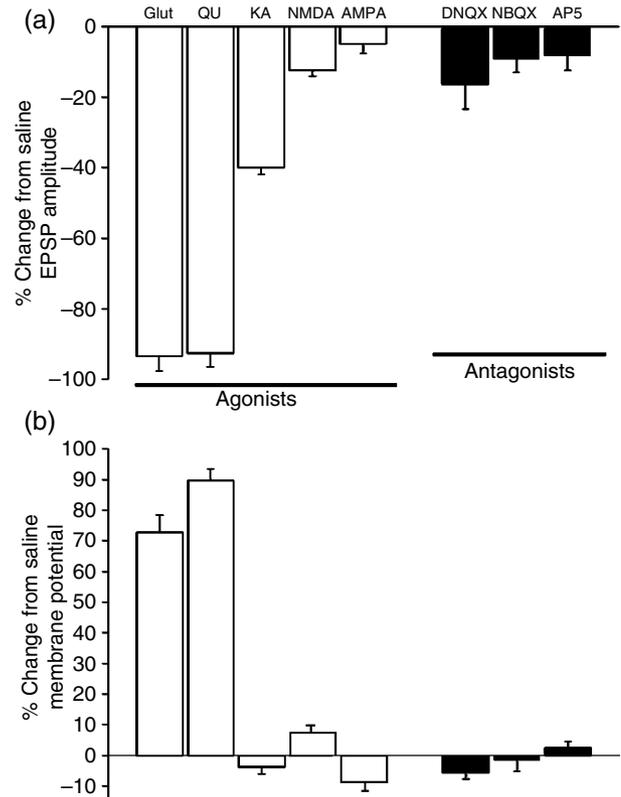
Fig. 2. The effects of agonists and antagonists on the compound Ib and Is excitatory postsynaptic potential amplitude (A) and the membrane potential (B) of the m6 muscle fibre. Five to six discrete preparations were used for each compound tested. A percentage change was determined within each preparation and these values were used for calculating mean ± SE. KA, Kainate ; Quis, quisqualate ; Glut, L-glutamate.
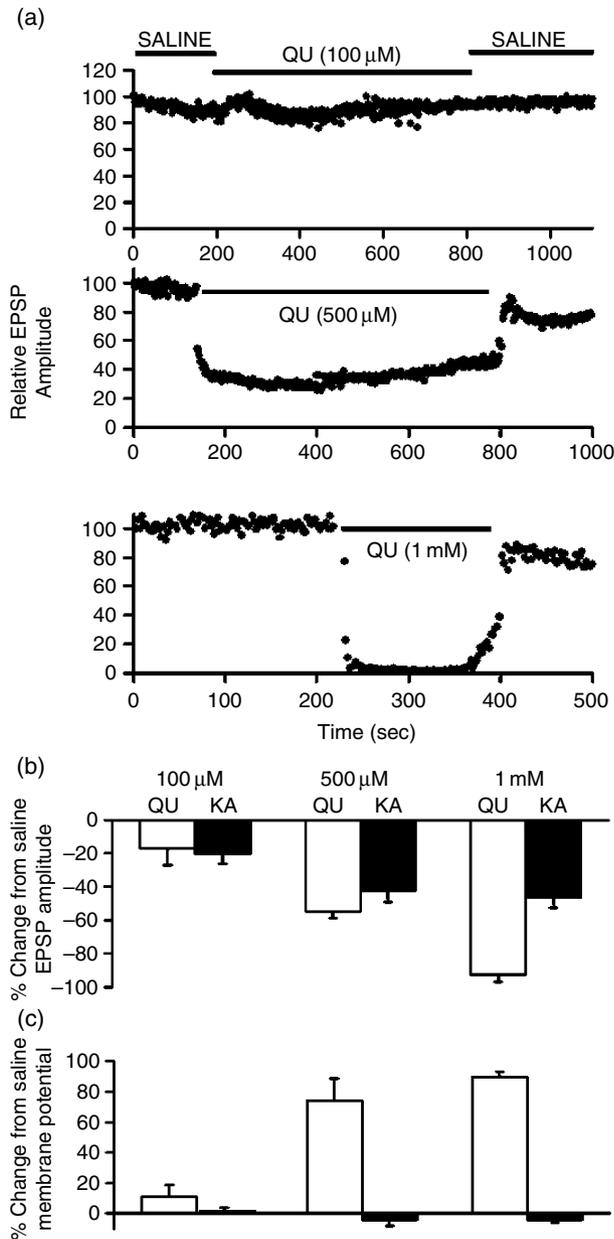
Fig. 3. Relative excitatory postsynaptic potential (EPSP) amplitude in the presence of 100 μM, 500 μM and 1 mM quisqualate. A relative change was determined within each preparation when considering the initial EPSP amplitude at 100% (A). The percent change in at least five preparations for each concentration of quisqualate and kainate was used to calculate the mean ± SEM effect on the compound EPSP amplitude (B) and the muscle fibre membrane potential (C). There is a clear dose–response effect for quisqualate on the EPSP amplitude and membrane potential. Kainate showed no significant effect on membrane potential; however, it did reduce the EPSP amplitude in a dose-dependent manner.

On comparing the dose–response effects for the two agonists, it is readily apparent that quisqualate has a greater effect at equivalent concentrations (Fig. 3B, $P < 0.05$

parametric, at each concentration). It is also interesting to investigate whether a concentration effect exists in altering the membrane potential of the muscle fibre. Only quisqualate shows an effect in depolarizing the membrane potential and this effect is dose-dependent (Fig. 3C). Along with the strong depolarizations of the fibres, the muscles are in a contracted state. With the substantial depolarizations, the EPSP amplitudes are masked and thus are not observed for measurement.

It is surprising that DNQX does not have a strong effect in reducing the evoked EPSP amplitude because it is a very potent antagonist for quisqualate receptors in vertebrates. If the synaptic receptors are indeed quisqualate, DNQX at 1 mM should substantially reduce the EPSP amplitude. The results suggest that there may be extrasynaptic quisqualate receptors on the muscle fibre and that those at the synapse might not be truly of a quisqualate subtype. To examine this possibility, different concentrations of DNQX and quisqualate are studied in combination. There is no significant differences in the EPSP reduction for 500 μM and 1 mM DNQX (Fig. 4). In addition, DNQX at 500 μM and 1 mM do not show any significant effect with respect to application of quisqualate at 500 μM. The effect of quisqualate in the presence of DNQX is the same as that without DNQX being present (Fig. 3B).

Because this synaptic model system, with a known genome, is so well utilized, it is surprising that this is the first study to report screening the commonly used pharmacological agents of vertebrates for molecular characterized glutamate receptors. Previously, Jan & Jan (1976) showed that *Drosophila* larval muscle was sensitive to L-glutamate and not sensitive to D-glutamate, L-glutamine, L-aspartate, L-asparagine, glycine, L-glutamine, and L-aspartate up to concentrations of 5 mM. They also showed that ACh and GABA (10 mM) had no effect an the NMJ or muscle. Later, Delgado *et al.* (1989) demonstrated the sensitivity of
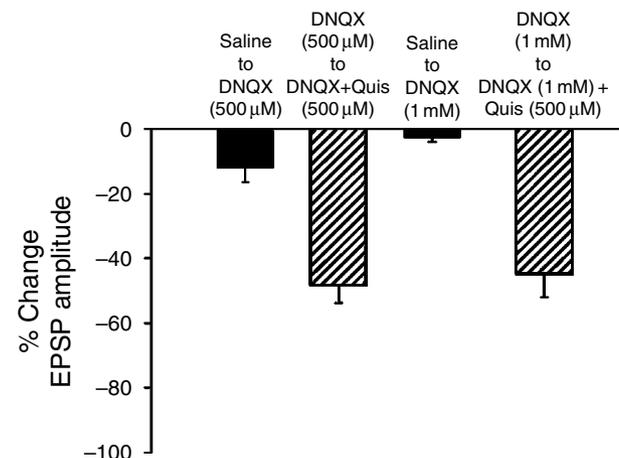
Fig. 4. The antagonist DNQX for quisqualate-sensitive receptors in vertebrates does not block the action of exogenous application of quisqualate at 500 μM or 1 mM. In addition, DNQX at 500 μM or 1 mM dose not effectively block evoked synaptic transmission.

Drosophila larval muscle to glutamate but noted some repolarization effect of the muscle for glutamate higher than 20 μM. The present study only covers depolarization effects with glutamate within the range of 100 μM to 1 mM. Delgado *et al.* (1989) also noted some anion-selective effect induced by GABA as well as by glutamate. These earlier studies have been extended to include the well-established vertebrate agonist and antagonists at this NMJ, as well as conducting the physiology in a saline more in keeping with the ionic concentrations known for *Drosophila* haemolymph. Because argiotoxin, a blocker of these glutamate receptors, is no longer commercially available, other pharmacological means are used to aid in characterizing these receptors.

The results imply that there are receptors on the surface of the muscle fibres that are very sensitive to glutamate and quisqualate. The rapid and prolonged depolarization produced by these compounds indicates a direct action on the muscle fibres, as was previously shown in patch clamp studies of muscle fibres (Chang & Kidokoro, 1996). The present study indicates that the antagonists do not have a role on the synaptic receptors; however, they could have an action on extrasynaptic receptors, which are not addressed here. The agonists and anatagonists could also play a role in targeting presynaptic autoreceptors on the motor nerve terminals. No gradual effects are noted for the various antagonists indicating that they do not have any presynaptic actions over time modulating evoked release. Because they demonstrate a small effect, if any, for such large concentrations (1 mM), it is unlikely they will be of pharmacological use in fully blocking synaptic responses at these NMJs. It would also be interesting to know whether the Ib and Is NMJs respond equally to the compounds examined. The dually innervated muscle in *Drosophila m.* as well as other fly species, such as *Megaselia scalaris* (Harrison & Cooper, 2003) and *Calliphora erythrocephala* (Hardie, 1976), offers a unique opportunity to examine mechanisms in synaptic differentiation on a single target by pharmacological and genetic approaches.

As noted previously by McLarnon & Quastel (1988), as well as in the present study, an unexpected finding is that kainate, a potent agonist for some vertebrate metabotropic receptors, does not produce a depolarization of the muscle fibre but does show a dose-dependent effect in reducing the EPSP amplitude. Thus, kainate could be acting as a post-synaptic antagonist directly on synaptic receptors with a relatively low affinity, or possibly acting presynaptically in reducing evoked transmitter release. By examining the frequency of release, the actions of kainate could be resolved, as attempted for the mGluRs (Zhang *et al.*, 1999). There is supportive evidence for a presynaptic action in kainate altering transmitter release from presynaptic terminals in vertebrates (Kamiya & Ozawa, 2000; Kamiya *et al.*, 2002). The mechanism of action is thought to be via kainate-sensitive glutamatergic autoreceptors within the presynaptic terminals; however, the detailed mechanisms remain unresolved. Possible scenarios have been proposed from kainate directly acting on the voltage-gated Ca^{2+} channel

or though a metabotropic action. Current studies employing quantal analysis are in progress to address the actions of kainate on nerve-evoked release and direct actions on the muscle receptors. In addition, voltage-clamping of the muscle fibres is now being used to delineate pre- and post-synaptic actions.

The potent DNQX antagonist for quisqualate-sensitive receptors in vertebrates apparently has no action in blocking the exogenous application of quisqualate at 1 : 1 and 1 (quisqualate) : 2 (DNQX) ratios. In addition, DNQX does not block evoked transmission. Thus, whether the synaptic receptors are truly a quisqualate subtype, because the effects reported might be mediated by extrasynaptic receptors that are also insensitive to DNQX, remains unknown. It is plausible that the affinity of the vertebrate anatagonists is substantially different in this invertebrate model. In the present study, the physiological profiles are highlighted for the known pharmacological agents in the hope of encouraging further studies to establish affinity-binding constants for these common agents and promote comparison of the molecular binding sites on the receptors. The sequences of these receptors are known in *Drosophila* muscle (Betz *et al.*, 1993; Völkner *et al.*, 2000; Marrus *et al.*, 2004).

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