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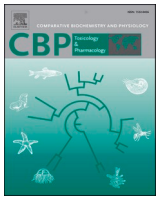

Research article

Fluoxetine antagonizes the acute response of LPS: Blocks K2P channels

Elizabeth R. Elliott, Robin L. Cooper *

Department of Biology, University of Kentucky, Lexington 40506, KY, USA

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ABSTRACT

The channels responsible for maintaining resting membrane potential are known as K2P (two-P-domain K^+ subunit) channels, a subset of which are known to be blocked by Fluoxetine. In this experiment, the compound's effects on the membrane potential were examined on muscles in larval *Drosophila* overexpressing a subtype of K2P channel (known in *Drosophila* as dORKA1 or ORKA1) and compared to larvae without overexpression. The compound was also observed in sequence and/or combination with a form of lipopolysaccharide (LPS) that transiently activates K2P channels. Different concentrations of Fluoxetine were tested, and it was also examined in cocktail with the LPS. At 25 μM Fluoxetine exposure, muscle in control larvae underwent depolarization, while muscles overexpressing K2P channels hyperpolarized; at 50 μM, however, much more variable responses were observed. The LPS caused hyperpolarization in both larval strains, but the effect was more transient in the Canton-S line than in the K2P overexpressors. Finally, LPS continued to cause hyperpolarization even in the presence of Fluoxetine, while Fluoxetine quickly depolarized the muscle during exposure to LPS. The cocktail showed a smaller effect on muscles overexpressing ORKA1 as compared to the controls, indicating that Fluoxetine does not block the ORKA1 subtype. This study is significant because it demonstrates how overexpression of K2P channels alters membrane response to LPS and Fluoxetine exposure.

1. Introduction

Two-P-domain K^+ subunit channels, or K2P channels, represent a subset of potassium ion leak channels known to exist in a wide array of organisms and with a variety of subtypes (Lesage et al., 1996a; Plant and Goldstein, 2015). These channels play a role in the maintenance of resting membrane potential, as well as the response observed when external K^+ concentration is altered (Nernst, 1888, 1889; Goldman, 1943; Hodgkin and Huxley, 1952; Hodgkin et al., 1952; Hodgkin and Katz, 1949; reviewed in: Hille, 1992); however, while many of these subtypes have been characterized according to their molecular sequence and their sensitivities to pH, mechanical stress, and pharmacological agents, much remains to be learned about them. A great deal of research is currently available on the K2P channel subtypes and how they function (Goldstein et al., 1998; Ilan and Goldstein, 2001; Kim, 2005; Enyedi and Czirják, 2010; Mathie et al., 2010; Noël et al., 2011; Lesage and Barhanin, 2011; Kuang et al., 2015; Feliciangeli et al., 2015; Plant and Goldstein, 2015; Kamuene et al., 2021).

The channel naming conventions relate to their function and sequence. The earliest defined TWIK protein (tandem pore domains in a weak inward rectifying K channel: TWIK-1) resulted the widespread adoption of this naming scheme, leading to such channels as the TWIK-related acid sensitive K+ channel (TASK) and TWIK-related K+ channel (TREK) (Kamuene et al., 2021). K2P channels have been identified in *Drosophila melanogaster* as K2PØ, KCNKØ, or dORK channels (Goldstein et al., 1996; Lesage et al., 1996; Lalevée et al., 2006), and these dORK channels are sequentially related to the human K2P channel subtype TREK, including a pH sensitivity similar to that observed in TASK subtypes (Duprat et al., 1997; Mukherjee and Sikdar, 2021). The body wall muscle of the larval *Drosophila* depolarizes in acidic conditions and recovers upon return to physiological pH, indicating possible expression of a TASK-like K2P channel (Badre et al., 2005; Cooper and Krall, 2022). The dORKA1 channel is referred to as ORK1 throughout this report.

The pharmacological profile, sensitivity to mechanical stress, and response to pH alterations of the ORK1 channel and accessory proteins have yet to be fully explored (Niemeyer et al., 2016; Zilberberg et al., 2000; Mazella et al., 2010). Different K2P channels exhibit different responses to selective serotonin reuptake inhibitors (SSRIs) (Dong et al., 2015; Djillani et al., 2019a) and, since TREK subtypes have been related to various disease states, it is important to gain better understanding of their function and response to therapeutic compounds (Djillani et al., 2019b). This could be achieved by investigating channel expression in

* Corresponding author.
E-mail addresses: erel222@uky.edu (E.R. Elliott), RLCOOP1@email.uky.edu (R.L. Cooper).

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various model systems, as well as by examining responses to such compounds.

It was recently demonstrated that therapeutic respiratory aid doxapram likely blocks ORK1 channels in larval *Drosophila* skeletal muscle, producing similar depolarization to that observed under acidic conditions (Vacassenno et al., 2023a, 2023b). Investigations into doxapram show that the drug has a larger effect in preparations with overexpression of ORK1 K2P channels, suggesting that they are involved in its mechanism of action (Elliott et al., 2024). Doxapram has also been investigated in connection with the effects of lipopolysaccharides (LPS) produced by Gram-negative bacteria. LPS from *Serratia marcescens* induces a rapid hyperpolarization of larval *Drosophila* muscle via activation of a K2P channel subtype (Cooper et al., 2019; Cooper and Krall, 2022; Vacassenno et al., 2023a, 2023b) and acts antagonistically towards doxapram's actions in both *Drosophila* and crayfish (Elliott et al., 2024, Brock and Cooper, 2023).

While the precise K2P channel subtypes present in larval *Drosophila* body wall muscles are not known, a TASK-like K2P channel is likely involved (Vacassenno et al., 2023a, 2023b; Elliott et al., 2024). It would thus be useful to investigate whether other K2P channel blockers with similar profiles to doxapram might result in similar physiological effects. Past experiments with the drug fluoxetine and its metabolite, norfluoxetine, suggest that they block K2P channels (Djillani et al., 2019a, 2019b; Proks et al., 2021), though it has also been observed to block voltage-gated sodium channels in neurons (Majeed et al., 2015). Additionally, given the aforementioned observation that greater ORK1 expression results in greater sensitivity to doxapram exposure, it would also be worthwhile to investigate how fluoxetine acts on tissue with an overexpressing of the known ORK1 K2P channel subtype.

It was demonstrated in 2019 that larval *Drosophila* muscle exposed to LPS sourced from particular forms of Gram-negative bacteria underwent a transient hyperpolarization of the muscle in a dose-dependent manner (Cooper et al., 2019). Since then, the mechanism by which LPS induces this rapid response has been linked to activation of a K2P channel subtype (Ballinger-Boone et al., 2020; Cooper and Krall, 2022; Brock and Cooper 2023; Elliott et al., 2024). LPS action can be dampened by doxapram exposure, acidic conditions, and, as demonstrated herein, ORK1 overexpression, since the membrane is already hyperpolarized due to the abnormally high presence of K2P channels. Since fluoxetine blocks K2P channels and LPS activates them, it would be useful to establish whether the two compounds also act in an antagonistic manner (i.e., whether fluoxetine exposure blocks action by LPS), so this study aimed to examine this relationship.

2. Methods

2.1. Animals

Drosophila melanogaster Canton S (CS) flies were used in physiological assays. This strain has been isogenic in the laboratory for several years after originally being obtained from the Bloomington Drosophila Stock Center (BDSC). Early third-instar *Drosophila* CS larvae were used (50–70 h) post-hatching. The CS larvae were maintained at room temperature, ~21 °C, in vials partially filled with a cornmeal-agar-dextrose-yeast medium. Overexpression of the dORK1 protein in larval body wall muscles (m6 and m7) was achieved by crossing homozygous males of.

P{w[+mW.hs] = GawB}BG487 (BDSC stock # 51634) with female virgins of UAS-ORK1 (BDSC stock # 6586; y^1 w*; P{w[+mC] = UAS-Ork1.Delta-C}2)(Nitabach et al., 2002). Progeny carrying one copy each of GAL4 driver and UAS-ORK1, referred to as body muscle M6-M7 > ORK1, were used for physiological analyses. *BG487-Gal4* expression pattern occurs as an anteroposterior gradient in larval body wall muscles 6/7. This allows BG487 to drive UAS–ORK1 specifically in muscles 6 and 7. (Budnik et al., 1996; Sulkowski et al., 2014). CS larvae were used for control comparisons.

2.2. Larval neuromuscular junction

The early third-instar larval body wall muscle m6 was used to monitor transmembrane potentials via sharp intracellular electrodes (30 to 40 MΩ resistance) filled with 3 M KCl. An Axonclamp 2B (Molecular Devices, Sunnyvale, CA, USA) amplifier and 1 X LU head stage was used. The membrane potential and spontaneous excitatory junction potentials (mEJPs) were collected and analyzed with LabChart 7.0 (ADInstruments, USA).

The larval dissections were performed as described in Cooper et al. (1995). In brief, a longitudinal mid-dorsal incision was made, and the four corners held in place with pins such that the preparation was stretched out along a glass slide. This preparation dish was originally described in studies of the leech nervous system (Muller et al., 1981). Internal organs were carefully removed to expose the body wall muscles, particularly the ventral longitudinal muscles of segment 3 (Fig. 1A). The brain and the segmental roots lie on the body wall muscles. To transect the nerve roots near the brain (thus affording the longest roots possible without damaging muscles in segments 1 and 2), the brain was lifted up via the gastrointestinal tract and draped over the dissection pin (Fig. 1B).

Medium exchange resulted in small deflections in the recordings in some cases, although these were generally observed only during the exchange itself and can thus easily be noted in the recording as artifactual. The use of an agar bridge with a chlorided silver wire placed inside the agar as a ground minimized the electrical defections from changing the media. A 1 % agar plug made in saline was constructed within a 200 μL Eppendorf pipette tip. The plastic pipette tip reduced the electrical offset from varied medium levels during bath exchanges, as the agar plug remains in contact with the bath for a given surface area independent of the level of the bathing solution.

Three experimental paradigms were used to investigate the effects of fluoxetine on larval muscle. One allowed observation of fluoxetine's direct action on membrane potential. The second focused on fluoxetine's action during exposure to LPS. The third focused on whether, in a preparation already exposed to fluoxetine, LPS could counteract the depolarization caused by fluoxetine, and vice versa. The direct effects of LPS were examined at the same concentrations to allow comparison against the action of fluoxetine and LPS combined. Comparisons were also drawn between responses observed in the CS strain versus the overexpressed K2P strain M6-M7 > ORK1 throughout the investigations.

Use of the genetically amenable *Drosophila* model allowed for selective altered expression of known K2P channel ORK1 in a subset of larval body wall muscles known as m6 and m7. The genetically modified Gal4 strain endogenously expresses green fluorescent protein (GFP)

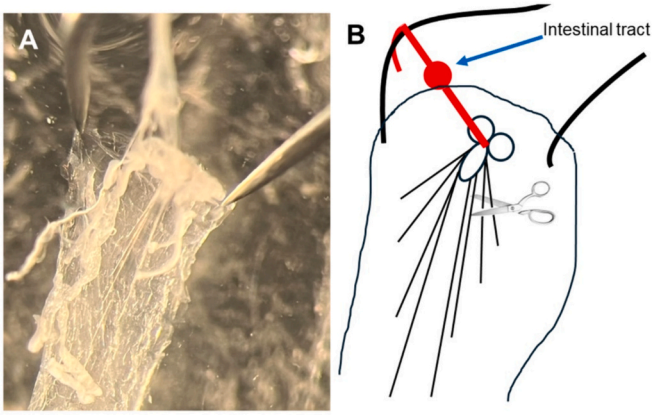

Fig. 1. Dissection of the *Drosophila* larvae in photo (A) and diagrammatic (B) form. The process of transecting the nerve roots near the brain is demonstrated using manipulation of the gastrointestinal tract to carefully expose the nerve roots for transection.

(Nitabach et al., 2002; Budnik et al., 1996; Sulkowski et al., 2014) such that, when crossed with the UAS-ORK1, both GFP and ORK1 are expressed in the pertinent cells, allowing for confirmation that the cross occurred successfully (see Budnik et al., 1996 for BG487 x UAS-LacZ with anti-βgal antibodies selective staining for cells in anteroposterior gradient). Segment 1 has a unique muscle (i.e., m31) with a very high expression of the GFP marker, but this cell is easily damaged during larval dissection; the m6 muscle in segment 2, however, is reliably preserved during dissection for use in physiological studies and so it was used in these studies. The GFP expression is shown in Fig. 2 for an early 3rd instar of M6-M7 > ORK1 strain.

2.3. Chemicals

The saline used was haemolymph-like fly saline 3(HL3) (de Castro et al., 2014; Stewart et al., 1994): (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 *N*, *N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) and pH at 7.2. Fluoxetine solutions were produced by dissolving Fluoxetine hydrochloride (SIGMA catalog # PHR1394) directly into the saline. Concentrated stocks were used for dilutions and for the cocktail with LPS. LPS from *Serratia marcescens* was dissolved in physiological saline on the day of experimentation. A high concentration of LPS (500 μg/mL) was used for comparison with previous studies to best address the mechanisms of action (Potter et al., 2021; Vacassenno et al., 2023b). In most paradigms,

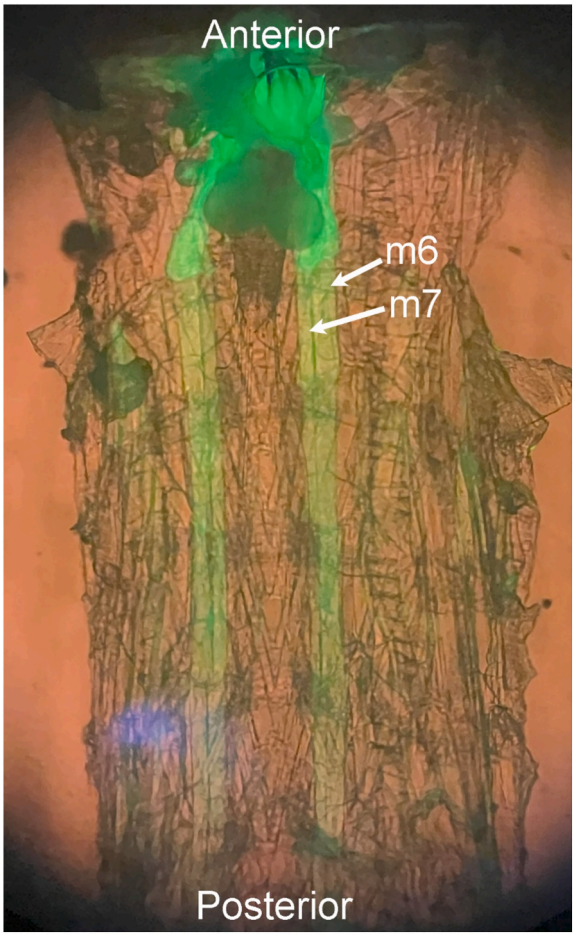

Fig. 2. The expression of GFP in the *BG487-Gal4* expression pattern. There is an anteroposterior gradient in larval body wall muscles 6/7. This allows BG487 to drive UAS–ORK1 specifically in muscles 6 and 7. The electrophysiological recordings were obtained in segment 2 of m6 for both M6-M7 > ORK1 and CS strains for response comparison.

Fluoxetine hydrochloride was applied at concentrations of 25 μM or 50 μM for 3 min at a time. The specific durations of exposure to Fluoxetine or LPS are shown in the scale bars within each paradigm's figure.

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Vigorous vortexing was required to dissolve LPS as well as possible. The pH of all solutions was maintained at 7.2 the day of experiments. The strong buffer of 25 mM BES in the saline helped to prevent drift in pH.

3. Statistical analysis

Analysis was performed using SigmaStat software. P of <0.05 was considered statistically significant. Paired and unpaired *t*-tests or Sign tests were used for statistical analysis. Paired t-tests were used for within-subject changes, and unpaired tests for comparisons between larval strains. All averaged data are expressed as means (±SEM). Percent change was determined as [(initial – condition examined)/ (initial)] *100.

4. Results

4.1. The effect of fluoxetine on membrane potential

Representative traces of the membrane potential in m6 of CS and M6-M7 > ORK1 larvae when exposed to Fluoxetine at 25 μM (Figs. 3A1 and B1) and 50 μM (Figs. 3A2 and B2) are shown. Fluoxetine at 25 μM produced a larger depolarization in controls (CS larvae) than the over-expressing K2P strain M6-M7 > ORK1 after three minutes of exposure (Figs. 3C1 and C2). At 50 μM, the CS strain showed varied responses, with some preparations depolarizing and others hyperpolarizing (Figs. 3D1 and D2). The CS strain exhibited a much larger depolarization at 50 μM than at 25 μM (*t*-test; $p < 0.05$), both in terms of raw membrane potential and percent change (taken from the initial response upon Fluoxetine exposure). Due to the high response variation observed with exposure to 50 μM, the remaining investigation used 25 μM Fluoxetine to examine the effects of additional exposure to LPS.

4.2. The direct effect of LPS on membrane potential

LPS exposure resulted in a rapid hyperpolarization of the membrane potential in both CS and M6-M7 > ORK1 strains, as shown in the representative traces (Fig. 4A and B). However, in M6-M7 > ORK1 larvae, LPS resulted in a prolonged hyperpolarization and did not significantly depolarize over time as is observed in the CS strain (Fig. 4C). Only upon washing out the LPS with fresh saline at least twice did the membrane potentials approach the initial values observed for both CS and M6-M7 > ORK1 strains (Fig. 4C). The percent change observed during hyperpolarization was not as large in the M6-M7 > ORK1 strain as in CS, likely due to the fact that the overexpressor line has a resting membrane potential closer to E_K than that observed in CS larvae (Fig. 4D). The percent change observed after three minutes of depolarization was substantially larger in CS larvae (*t*-test, $p < 0.05$; Fig. 4D).

4.3. The effect of fluoxetine followed by the acute action of LPS in the presence of fluoxetine

Since exposure to LPS resulted in rapid hyperpolarization, presumably through activation of a K2P channel subtype present in larval CS muscle, it was of interest to investigate the possibility that Fluoxetine blocks or retards this acute response. To do so, the preparations were exposed to a Fluoxetine solution absent LPS, after which the solution was replaced with one containing both Fluoxetine (at the same concentration) and LPS to ensure LPS exposure without Fluoxetine removal. Since M6-M7 > ORK did not undergo strong depolarization under Fluoxetine exposure but did exhibit prolonged hyperpolarization with

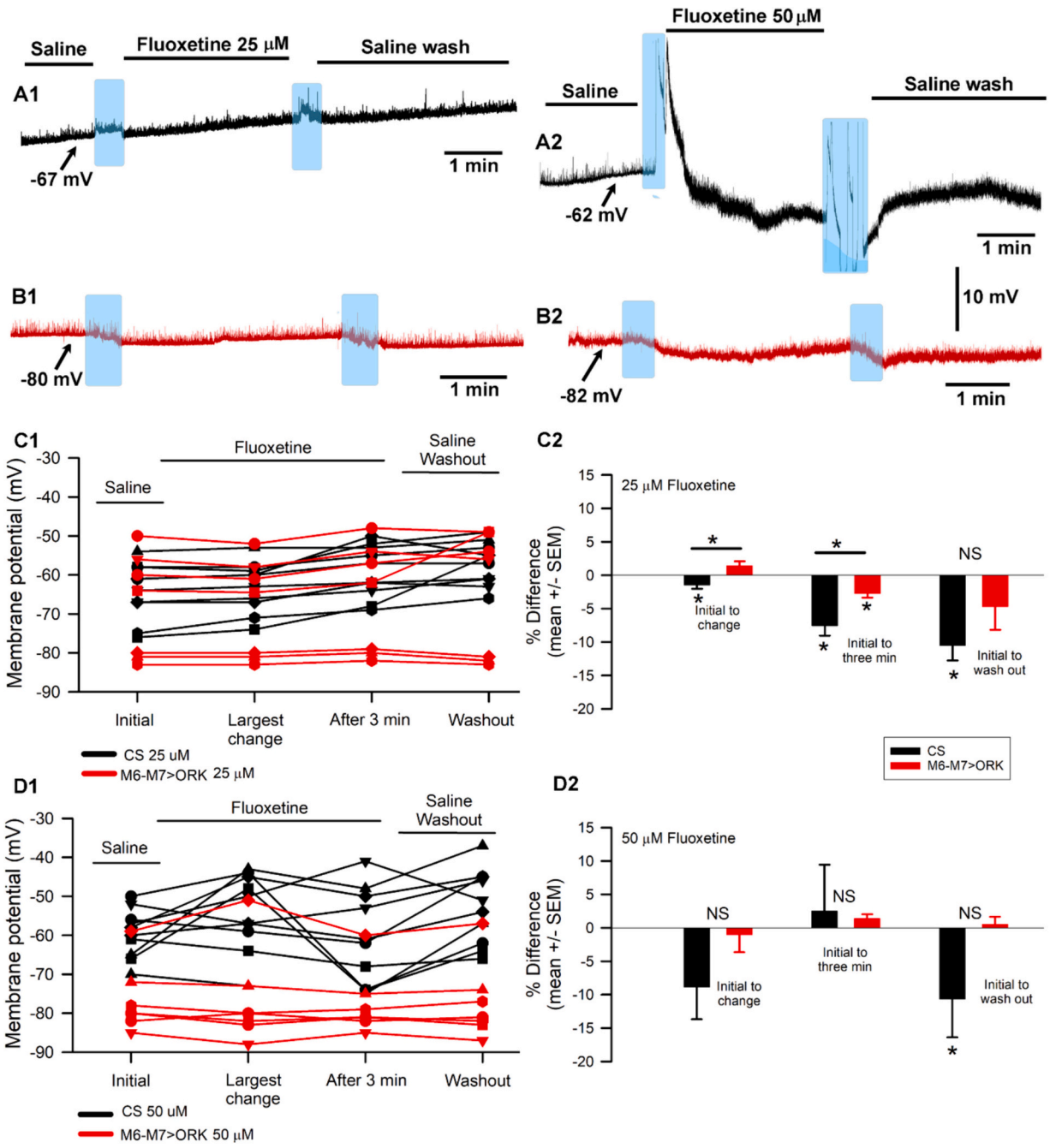

Fig. 3. The acute effects of Fluoxetine on cell membrane potential in organisms with and without ORK1-K2P overexpression. Representative traces of membrane potentials are shown for CS (A1, A2) and M6-M7 > ORK (B1, B2) strains under exposure to Fluoxetine at 25 μM (A1, B1) and 50 μM (A2, B2). The light blue shadow boxes depict when the saline bath was switched. The responses for individual preparations are shown under exposure to 25 μM (C1) or 50 μM (D1) in both CS (black lines) and M6-M7 > ORK (red lines). The mean (+/− SEM) normalized percent changes from each individual preparation's initial value to that under each condition (from initial to the largest potential change, potential after three minutes of Fluoxetine exposure, and potential from two minutes after wash-out) are shown for 25 μM (C2) or 50 μM (D2) in both CS (black) and M6-M7 > ORK (red) lines. Each washout featured two exchanges of the bath for fresh saline. The asterisk (*) by each individual bar represents a significant difference (paired *t*-test; $p < 0.05$) in the change from initial saline to the response (within a single strain). The asterisk-line pairing (*) spanning both bars represents a significant percent change between the CS and the M6-M7 > ORK strains (t-test; $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LPS, if was also of interest to know whether fluoxetine would produce some depolarization over time when exposed to the cocktail.

However, this was not the case. Representative traces for both CS and the M6-M7 > ORK larvae are shown (Fig. 5A and B, respectively), and the response trends were generally consistent across both *Drosophila* strains (Fig. 5C). The CS strain underwent a marked hyperpolarization followed by a depolarization, which is a response significantly different from that observed in the M6-M7 > ORK strain (Fig. 5D). M6-M7 > ORK demonstrated a consistent hyperpolarization during both exposure to the LPS-fluoxetine cocktail and washout (paired *t*-test, $p < 0.5$; Fig. 5D), remaining significantly different from responses observed in CS larvae (t-test, $p < 0.5$; Fig. 5D).

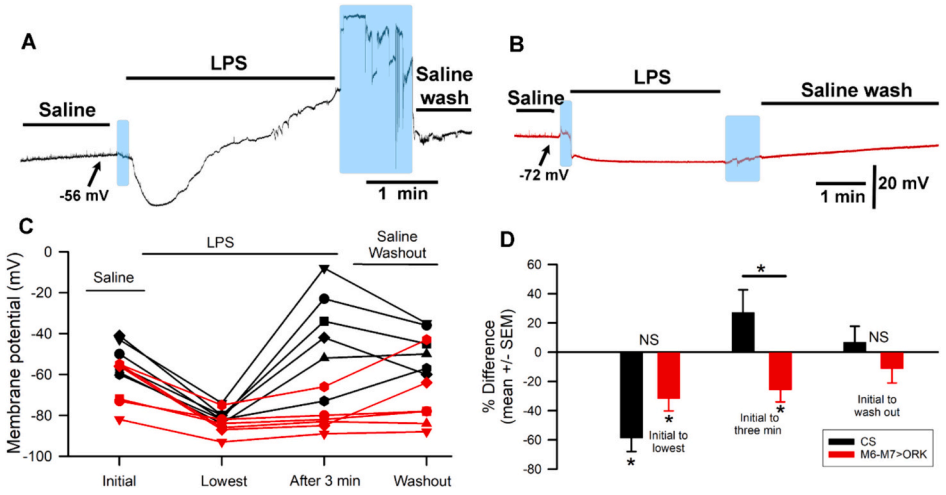

Fig. 4. The acute effects of LPS exposure on the resting membrane potential in CS and M6-M7 > ORK1. Representative responses for CS (A) and M6-M7 > ORK1 (B). Note the transient hyperpolarization observed in the CS strain as opposed to the persistent hyperpolarization in the M6-M7 > ORK1 strain. The light blue shadow boxes depict when the saline bath was switched. The responses for individual preparations of each strain are shown (C). The mean (+/− SEM) normalized percent changes from each individual preparation's initial value to that under each condition (from initial to the largest potential change under LPS exposure, potential after three minutes of LPS exposure, and potential from two minutes after wash-out) are shown for both CS (black) and M6-M7 > ORK (red) (D). Each washout featured two exchanges of the bath for fresh saline. The asterisk (*) by each individual bar represents a significant difference (paired *t*-test; $p > 0.05$) in the change from initial saline to the response (within a single strain). The asterisk-line pairing (*) spanning both bars represents a significant percent change between the CS and the M6-M7 > ORK strains (t-test; p > 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

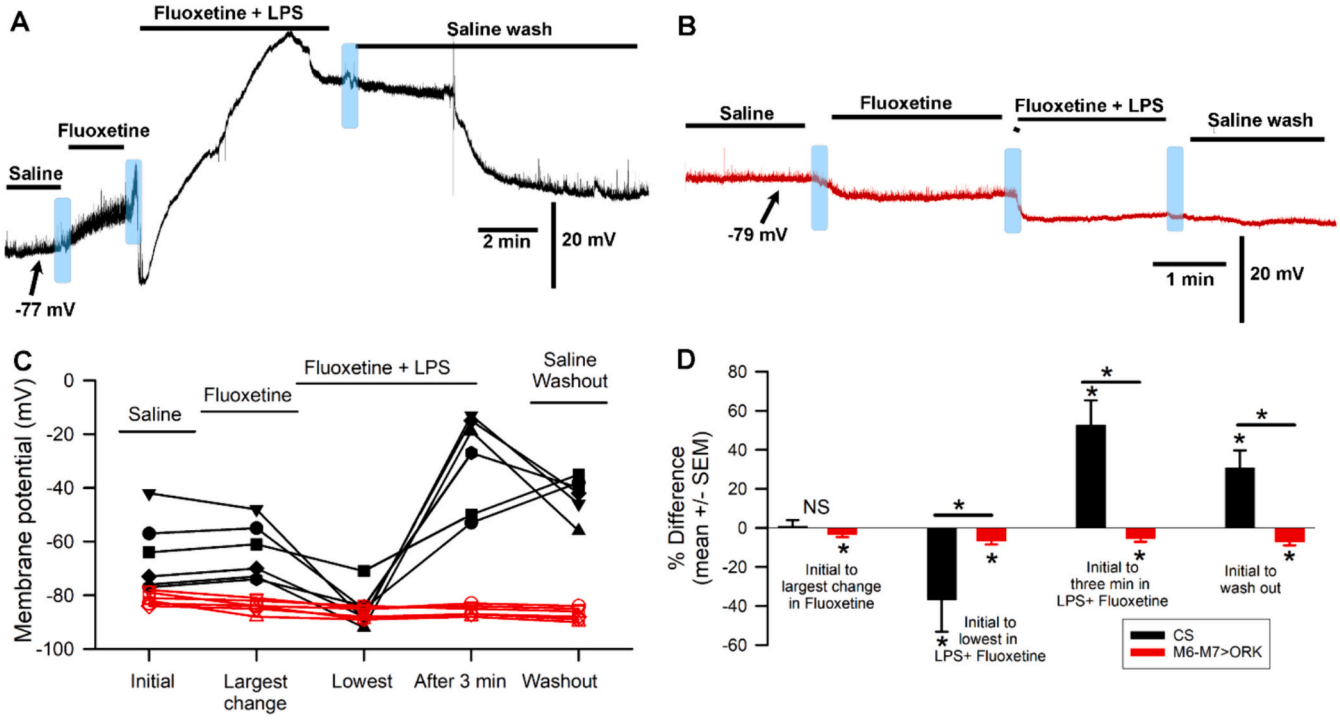

Fig. 5. The acute effects of exposure to Fluoxetine, followed by a Fluoxetine-LPS cocktail, on the resting membrane potential in CS and M6-M7 > ORK1. Representative responses for CS (A1) and M6-M7 > ORK1 (A2). Note the depolarization observed in the CS strain as opposed to the slight hyperpolarization in the M6-M7 > ORK1 strain during Fluoxetine-only exposure, as well as the fact that Fluoxetine-LPS cocktail exposure in the CS strain results in rapid hyperpolarization followed by depolarization, while the M6-M7 > ORK1 does not undergo depolarization. The light blue shadow boxes depict when the saline bath was switched. The responses for individual preparations of each strain are shown (C). The mean (+/− SEM) normalized percent changes from each individual preparation's initial value to that under each condition (from initial to the largest potential change under exposure to Fluoxetine, largest potential change immediately after exposure to the cocktail, potential after three minutes of exposure to the cocktail, and potential after washout) are shown for both CS (black) and M6-M7 > ORK (red) (D). Each washout featured two exchanges of the bath for fresh saline. The asterisk (*) by each individual bar represents a significant difference (paired *t*-test; $p < 0.05$) in the change from initial saline to the response (within a single strain). The asterisk-line pairing (*|) spanning both bars represents a significant percent change between the CS and the M6-M7 > ORK strains (t-test; p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.4. The effect of LPS followed by the acute action of fluoxetine in the presence of LPS

In CS larvae, exposure to LPS consistently led to a rapid hyperpolarization of the membrane, though the degree of that hyperpolarization was dependent on the initial resting membrane potential. The M6-M7 > ORK1 larvae, however, showed a reduced effect from LPS exposure, which is likely due to the membrane potential already being close to the equilibrium potential for K^+. The effects of LPS exposure did not seem to be altered by pre-existing exposure to Fluoxetine; however, that then raised the question of whether pre-exposure to LPS altered the effects of the Fluoxetine-LPS cocktail.

Representative responses for CS and M6-M7 > ORK1 are shown during both LPS exposure and subsequent exposure to the cocktail (Fig. 6A and B, respectively). Since exposure of CS larval muscle to LPS normally resulted in an acute hyperpolarization and, subsequently, a gradual depolarization, the initial exposure to LPS was kept short to ensure that the cocktail's effect could be assessed. Exposure to fluoxetine (via the cocktail) after LPS pre-exposure resulted in a rapid and pronounced depolarization in CS larvae (Paired *t*-test, $p < 0.5$; Fig. 6B and C) but not in the M6-M7 > ORK1 larvae, whereupon the response was similar to that previously observed under Fluoxetine pre-exposure: a consistent percent change indicative of slight hyperpolarization relative to the initial membrane potential (Paired t-test, $p < 0.5$; Fig. 6D).

Overall, the membrane potential of the M6-M7 > ORK1 strain was more negative that the CS strain, even when taking into account the potential variations observed among the individuals of each strain ($N = 38$ of CS, $N = 32$ of M6-M7 > ORK1; unpaired t-test $p < 0.001$), with a mean (+/− SEM) potential of −60.7 mV (±1.5) for CS and − 72.2 mV (+/− 1.8) for M6-M7 > ORK1. It thus appears as though overexpression of K2P channels may be driving the membrane potential to a more negative value for which the muscle does not compensate through altered expression of pumps or Na^+ leak channels (NALCN).

5. Discussion

This investigation illustrated that exposure of the *Drosophila* CS line to 25 μM Fluoxetine resulted in depolarization of the membrane, while exposure to 50 μM led both to muscle contractions that rendered it difficult to maintain an intracellular recording and to greatly varied responses, with some preparations undergoing large depolarizations and others hyperpolarizing. With overexpression of the ORK1 K2P channel subtype in larval muscle fibers, the resting membrane potentials were hyperpolarized and Fluoxetine had little by way of a depolarizing effect to the point that it failed to block the acute hyperpolarization that constitutes LPS action on membrane potentials.

Investigations into LPS exposure suggested that both strains underwent hyperpolarization but did so in different manners; the M6-M7 > ORK line produced prolonged hyperpolarization, while the CS strain underwent a more pronounced, transient hyperpolarization and then immediately entered a depolarization phase. In the CS strain, exposure to the LPS-Fluoxetine cocktail led to hyperpolarization after Fluoxetine pre-exposure and depolarization after LPS pre-exposure. The M6-M7 > ORK1 strain, on the other hand, had no significant response to the cocktail with LPS pre-exposure (though a slight hyperpolarization was observed), and exposure to the cocktail after Fluoxetine pre-exposure

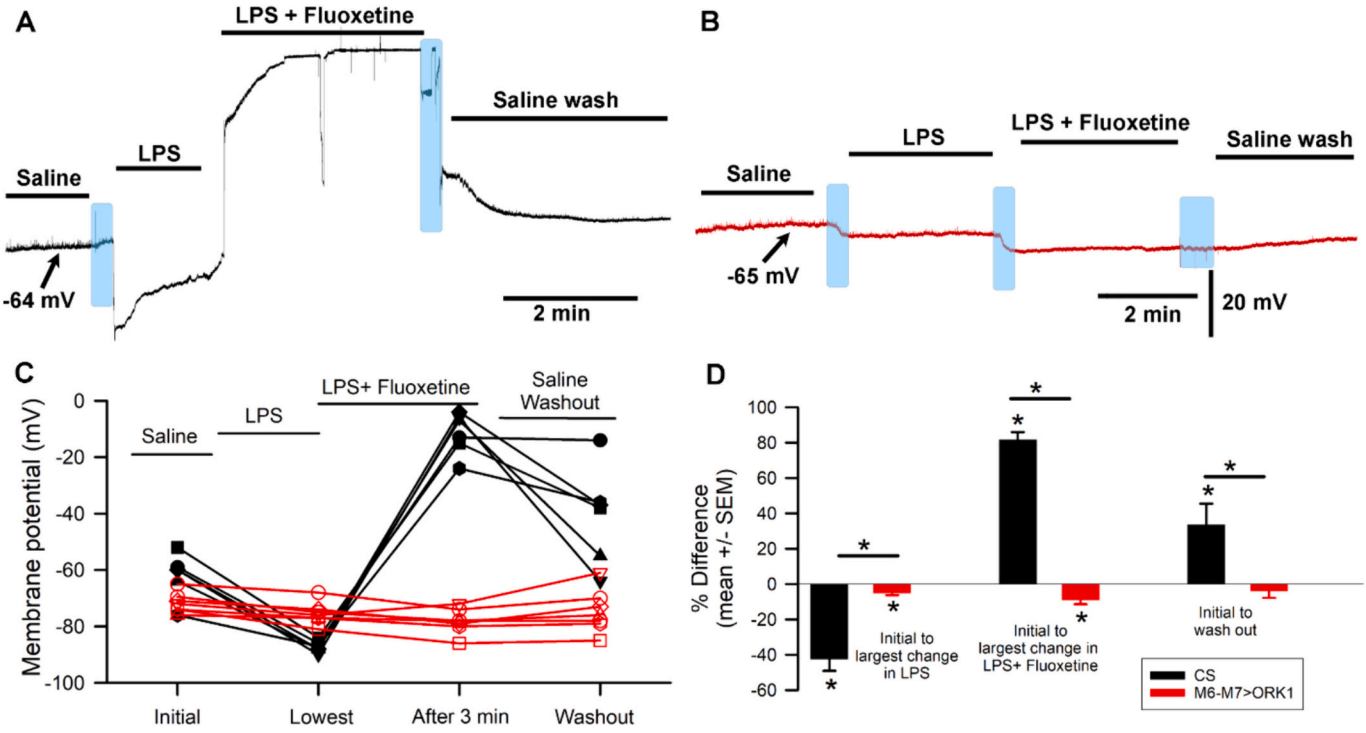

Fig. 6. The acute effects of exposure to LPS, followed by a Fluoxetine-LPS cocktail, on the resting membrane potential in CS and M6-M7 > ORK1. Representative responses for CS (A) and M6-M7 > ORK1 (B). Note the hyperpolarization observed in the CS strain as opposed to the slight hyperpolarization in the M6-M7 > ORK1 strain during LPS-only exposure, as well as the fact that Fluoxetine-LPS cocktail exposure in the CS strain results in rapid depolarization, while the M6-M7 > ORK1 does not undergo that depolarization. The light blue shadow boxes depict when the saline bath was switched. The responses for individual preparations in each strain are shown (C). The mean (+/− SEM) normalized percent changes from each individual preparation's initial value to that under each condition (from initial to the largest potential change under exposure to LPS, largest potential change after exposure to the cocktail, and potential after washout) are shown for both CS (black) and M6-M7 > ORK (red) (D). Each washout featured two exchanges of the bath for fresh saline. The asterisk (*) by each individual bar represents a significant difference (paired t-test; $p < 0.05$) in the change from initial saline to the response (within a single strain). The asterisk-line pairing (*) spanning both bars represents a significant percent change between the CS and the M6-M7 > ORK strains (t-test; $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

yielded similar results. It was interesting to note that LPS induced prolonged hyperpolarization in the K2P overexpressor line whether the exposure came from LPS alone or from the cocktail; this suggests that LPS transiently activates the K2P channels present in *Drosophila* CS muscle and activates (without desensitization) the ORK1 channels.

Fig. 7 summarizes the implications drawn from this investigation's results for the two strains (larval CS and M6-M7 > ORK1) with graphical depictions of a muscle cell from each line.

In CS preparations, the responses observed with exposure to 25 μM Fluoxetine may be due to inhibition of the endogenous K2P channels in the muscle. Exposure to Fluoxetine led to continual depolarization throughout the duration of exposure, suggesting that the responsive channels do not undergo desensitization, and there tended to be an increase in the frequency of spontaneous quantal events (miniature synaptic potentials) for some preparations; this would indicate that 25 μM Fluoxetine also affects motor neurons to promote these spontaneous events and that the depolarization observed in the muscle fibers is thus a response likely produced by a combination of pre- and post-synaptic contributions. Addressing the compound's effects on motor neurons is beyond the scope of the current study. In regards to the overexpressor line, since Fluoxetine and its metabolite, norfluoxetine, have been shown to block a selective subset of K2P channels known as TREK-2 (Djillani et al., 2019a, 2019b; Kennard et al., 2005; Proks et al., 2021), it is likely that a similar K2P subtype is expressed in larval muscle and motor neurons. However, the ORK1 channels overexpressed in the M6-M7 > ORK1 do not seem to be inhibited by 25 μM Fluoxetine, given that depolarization of the muscle was not observed.

The varied effects of the 50 μM Fluoxetine in both CS and M6-M7 > ORK1 muscle may be due to a lower affinity with other channels present in the muscle and motor nerve, such as voltage-gated Na^+ and/or Ca^{2+} channels. Exposure to Fluoxetine at concentration range 10–100 μM has been shown to block voltage-gated channels for the passage of Na^+ (Pancrazio et al., 1998), K^+ (Tytgat et al., 1997), and Cl^- (Maertens et al., 1999). Both smooth muscle voltage-gated K^+ channels and TREK-2 K2P channels are blocked, depending on the concentration (Farrugia, 1996; Kennard et al., 2005; Proks et al., 2021). Three subtypes of voltage-gated calcium channels (T-, N- and L-type) have been shown to undergo partial blockage (Deák et al., 2000); additionally, since the presynaptic voltage-gated calcium channels at the crayfish NMJ constitute P-type channels (Araque et al., 1994; Hong and Lnenicka, 1997) and crayfish motor neurons are affected, it is likely that Fluoxetine blocks P-type channels as well as voltage gated Na^+ channels (Majeed et al., 2015).

At the larval *Drosophila* motor nerve terminals, a Ca v2.1 subtype may also be blocked (Cunningham et al., 2022). The blockage of potential K2P channels by Fluoxetine (50 μM) in crayfish motor axons was also recently substantiated (Wang et al., 2024). Additionally, the fact that Fluoxetine exhibits a voltage-dependent blockage of TREK channels (Kennard et al., 2005; Proks et al., 2021) may explain some of the response variation observed in these data, since even muscle fibers alone exhibit different resting membrane potentials, whether within the *Drosophila* stains or between them. There has yet to be an analysis of the endogenous K2p channel subtypes present in the muscles or organelles of *Drosophila* skeletal muscles, and, while advancements with RNAseq may allow such discoveries to be forthcoming, such an endeavor is beyond the scope of this study.

Given that a voltage-gated K^+ channel was previously described in *Drosophila* body wall muscle as being blocked by tetraethylammonium chloride (TEA) (Gho and Mallart, 1986), our group has previously examined whether, respectively, TEA (20 mM), 4-aminopyridine (4-AP, 3 mM), and quinidine hydrochloride monohydrate (0.1 mM) could block LPS-induced transient hyperpolarization (Cooper et al., 2019; McCubbin et al., 2024); none of these potassium channel blockers inhibited LPS response. Additionally, RNAi lines and a mutation line constructed for the known *Drosophila* K(Ca) were investigated and had no effect on LPS response (Ballinger-Boone et al., 2020; McCubbin et al., 2024). It also appears unlikely that LPS activated the Na—K ATP pump or promoted Ca^{2+} influx, as the response was maintained even when preparations were acutely incubated with ouabain and ion substitution ($[Ca^{2+}]_o$ for $[Ba^{2+}]_o$, or $[Cd^{2+}]_o$, or $[Gd^{3+}]_o$) carried out (Potter et al., 2021; McCubbin et al., 2024).

The muscular effects of exposure to LPS from *Serratia marcescens* alone are intriguing, given that it has been postulated that the resulting hyperpolarization may stem from transient activation of K2P channels by the LPS (Cooper et al., 2019; Cooper and Krall, 2022; Elliott et al., 2024; Vacassenno et al., 2023a, 2023b). It is not yet understood why exposing the muscle to LPS at the concentrations used in this study results in gradual depolarization after the acute hyperpolarization until the potential rests above the initial RMP (Ballinger-Boone et al., 2020). Larval *Drosophila* muscle cells have not completely lost function even after depolarization to a membrane potential of almost zero (assessed within a short period, ~5 min, of LPS exposure). Flushing the

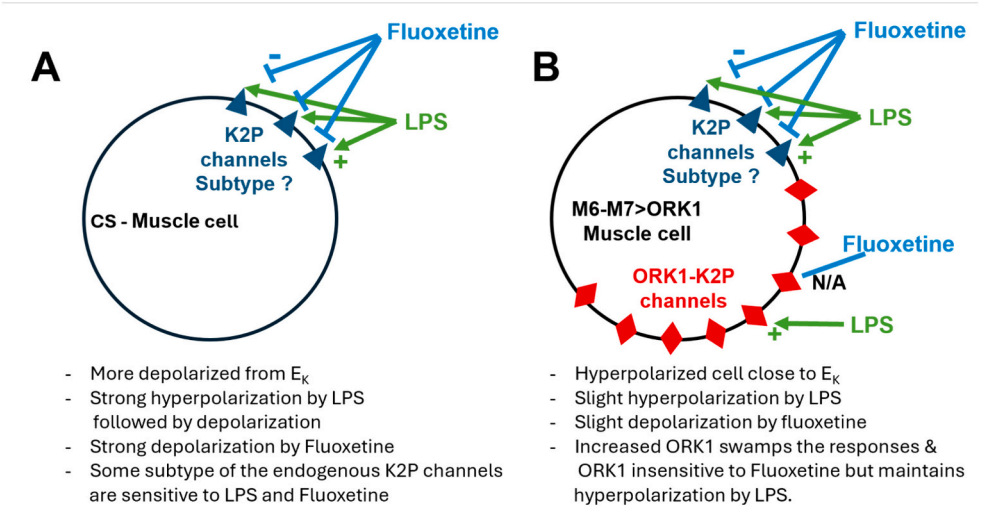

Fig. 7. Descriptive model to illustrate the effects of Fluoxetine on the CS strain as compared to a strain overexpressing the *Drosophila* K2P channel subtype dORK1. The endogenous expression of K2P channel subtypes in larval muscle is not fully characterized, but LPS and Fluoxetine both seem to affect at least one K2P channel subtype. When dORK1 is overexpressed, the effects of both LPS and Fluoxetine are masked by strong K^+ leakage through the dORK1 channels. Fluoxetine does not appear to affect dORK1 channels, but LPS has a prolonged effect on activating them, as the transient effect on the endogenously expressed K2P subtype is not present. (In the figure, *N/A* stands for "not applicable".)

preparation with fresh saline brings back membrane potential (Ballinger-Boone et al., 2020), and this same response can be observed again with another application of LPS, albeit with diminished responsiveness over time (Istas et al., 2020). This indicates that LPS can be washed off the muscle cell and thus isn't tightly bound, which in turn suggests that the application of a competitive LPS-receptor/channel inhibitor might dampen the effects of LPS.

Given that the hyperpolarization of *Drosophila* muscle does not appear to be mediated by the immune deficiency (IMD) signaling pathway responsible for initiating insect immune response to Gram-negative bacteria (Bangham et al., 2006; Lemaitre and Hoffmann, 2007; Aggarwal and Silverman, 2008; Hetru and Hoffmann, 2009; Ballinger-Boone et al., 2020), it appears as though a different mechanism is responsible for LPS binding to K2P channels. This novel mechanism of action may partly explain why the insect immune response is so complicated (Coscia et al., 2011; Loker et al., 2004). The possibility that LPS-induced hyperpolarization occurs due to acute K2P channel activation has yet to be confirmed at the single-channel level; similarly, the explanation for the transience of this response is not yet known either.

LPS may affect other channels in the muscle, such as voltage-gated Na^+ or Ca^{2+} channels or NALCN channels. The fact that larval CS muscle begins to contract in waves during LPS-induced depolarization suggests that Ca^{2+} may be increasing within the muscle cell. Since Ca^{2+} enters the muscle through voltage-sensitive Ca^{2+} channels in the plasma membrane, they could be opening due to the direct action of LPS or the indirect action of Na^+ entry bringing about depolarization. LPS also blocks postsynaptic glutamate receptors on the muscle of both larval *Drosophila* and crayfish, which can once again be reversed upon removal of the LPS (Ballinger-Boone et al., 2020; Cooper and Krall, 2022). The results reported herein suggest that the acute effects of LPS may depend on the K2P channel subtype being expressed, since dORK1 overexpression resulted in prolonged hyperpolarization rather than a transient response. If the depolarization is partly caused by action on other channels, this effect might be masked by the high expression of K2P dORK1 channels, but this topic has yet to be addressed.

Since it appears that LPS has an acute effect beyond that explained by the established genomic IMD-mediated pathways, it would be of interest to know whether compounds that might block LPS binding would affect either response. No known blockers to the insect IMD membrane receptor or the mammalian Toll-like receptor 4 (TLR4: a CD14/TLR4/MD2 complex) exist (Fitzgerald et al., 2003; Poltorak et al., 1998; Shimazu et al., 1999; Horng et al., 2001); however, if a fraction of the LPS response can be blocked, this might be beneficial to the overall therapeutic actions of an LPS surge treatment (which results in the lysing of bacteria) like that involved in the antibiotic treatment for septicemia. Since the initial immune response to LPS was discovered in the *Drosophila* model (Weiss and O'Neill, 2022), studying the acute effects of LPS on these channels might shed light on the diverse nature of its mechanisms of action.

K2p channel subtypes are highly varied, to the point that they have several distinct, selective blockers and activators (Buckingham et al., 2005; Duprat et al., 1997; Patel et al., 1999; Kim et al., 2000; Rajan et al., 2000; Kim, 2005; Plant and Goldstein, 2015; Kamuene et al., 2021). Doxapram appears to block the K2p channel subtype endogenously expressed in larval muscle (Vacassenno et al., 2023a, 2023b; Elliott et al., 2024), though this subtype has yet to be characterized at the molecular level. Additionally, Doxapram blocks the dORK1 subtype when it is overexpressed in *Drosophila* muscle (Elliott et al., 2024) and likely blocks the K2P channels endogenously expressed in *Drosophila* motor neurons, as the muscle depolarizes and evoked transmission occurs spontaneously (Elliott et al., 2024). As mentioned above, Fluoxetine was used in this study because it also appears to block a subtype of K2P channel (Dong et al., 2015; Djillani et al., 2019a, 2019b), but it appears to block neither the same subtypes that LPS activates nor the *Drosophila* specific ORK1 subtype.

Since certain mammalian pathologies result in altered K2P channel expression, including a wide variety of subtypes and highly varied pharmacological profiles (Lee et al., 2021; Wiedmann et al., 2021), and since LPS appears capable of opening some K2P channel subtypes, it is necessary to continue investigation into the properties of the subtypes themselves. It is yet unknown what effect may be caused by diverse channel expression levels in various tissues. To understand how K2P channels affect cellular properties and membrane potential variation among cells, research using cell culture, whole tissue, and various animal models will need to be continued. The *Drosophila* model offers easier genetic manipulation of protein expression on a tissue-by-tissue basis within intact animals, as well as enabling RNA knockdown to address functional significance (Ugur et al., 2016; Yamaguchi and Yoshida, 2018). This is accompanied by some side effects. When ORK1 channels were overexpressed in all muscles of the *Drosophila* larvae, an increase in lethality was observed (Elliott et al., 2024); when overexpressed in cardiac tissue, the preparations underwent abnormal cardiac function (Elliott et al., 2023). Other tissues within *Drosophila* have yet to be examined via experimental manipulations and/or the determination of endogenous K2P subtype expression levels, though recently developed approaches using 10× Genomics or BD Rhapsody for the examination of expression profiles in single cells may bolster this process (Gao et al., 2020).

In conclusion, the ORK1 channels genetically expressed in *Drosophila* body wall muscle were not blocked by Fluoxetine; likely, an as-of-yet unknown K2P channel subtype endogenously expressed in the muscle was blocked, hence the depolarization of the muscle observed and recorded herein. Additionally, the transient activation of K2P channels induced by LPS exposure was not blocked by Fluoxetine.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. The first draft of the manuscript was written in parts by all authors and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Elizabeth R. Elliott: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Robin L. Cooper:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data is prersented in manuscript

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