

1 **A non-invasive, fast on/off “Odourgenetic” Method to Manipulate Physiology**

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20 **Summary**

21 **Manipulating molecular processes governing physiological functions has significant**
22 **potential for clinical therapeutics and is an important approach to elucidate the cellular**
23 **basis of physiological functions. Here, we designed a Odourgenetic system co-expressed**
24 ***Drosophila* odorant receptor system (DORs) consisting of OR35a and OR83b, which were**
25 **exclusively activated by their odor ligand, 2-pentanone. Applying 2-pentanone to DOR-**
26 **expressing cells or tissues induced calcium influx and membrane depolarization. By**
27 **inhalation of 2-pentanone, we successfully applied DORs to manipulate behaviour, control**
28 **insulin secretion and regulate blood glucose and manipulate muscle contraction and**
29 **associated limb movement. Because 2-pentanone rapidly enters the blood upon inhalation**
30 **and leaves the body by exhalation, this odorant can be used with DORs to manipulate**
31 **cellular function, and the manipulation can be terminated at any time. Such feature**
32 **approach significantly improves the safety and controllability of DORs used in the clinic.**
33 **Thus, the present study developed a non-invasive, controllable, fast on/off method to**
34 **manipulate cellular activity and behaviour on a time scale of minutes.**

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37 Developing a rapid, controllable method for manipulating physiological functions has significant
38 potential for clinical therapeutics and basic research. Optogenetics has provided optical control
39 of neuronal activity at the millisecond time scale¹⁻⁵. However, this approach requires direct
40 optical access to brain tissue, which is difficult because blue light does not readily penetrate
41 whole organisms; light must be delivered using costly specialized equipment such as custom
42 blue light sources with fibre optics or two-photon illumination systems⁶. Additionally, given the
43 need for invasive equipment implantation and sufficient power⁷, it is difficult to apply
44 optogenetics for disease treatment in real clinical practice.

45 Chemogenetic designer receptors exclusively activated by designer drugs (DREADDs)⁸ are a
46 powerful approach for remote and transient manipulation of cellular activity with no need for
47 specialized equipment^{9,10}. A recent study showed that metabolically derived clozapine arising
48 from systemic clozapine N-oxide (CNO) administration is indeed the *in vivo* actuator of
49 DREADDs⁹. Clozapine binds with high affinity to many receptors and has side effects such as
50 behavioural inhibition and potentially fatal agranulocytosis¹¹. Thus, the use of clozapine as a
51 DREADD actuator in humans may result in undesirable side effects⁹. Converted clozapine
52 reaches its maximal concentration at 2–3 hours after CNO treatment¹², indicating that the effects
53 of CNO on cellular activity are most likely to occur at this time point. These dynamic
54 pharmacological profiles of CNO *in vivo* result in a long and uncontrollable process due to
55 irreversible drug effects and metabolism, which limits the potential for emergency clinical
56 applications such as seizure control¹⁰.

57 *Drosophila melanogaster* odorant receptor 35a (OR35a) belongs to the seven-transmembrane
58 G-protein-coupled receptor superfamily and is a typical ligand-gated ion channel¹³; this channel

59 forms a complex with the odorant-binding subunit odorant receptor 83b (OR83b)¹⁴⁻¹⁶. 2-
60 Pentanone is the natural ligand of OR35a. In the present study, we co-expressed OR35a and
61 OR83b in rodent tissues by viral transduction. We demonstrated that *Drosophila* odorant
62 receptors (DORs) were activated by inhalation of 2-pentanone and effectively manipulated
63 physiological processes and rodent behaviour on a time scale of minutes, indicating excellent
64 controllability of DORs in practice. Here, we provide an easy-to-use, noninvasive, and
65 spatiotemporally controllable approach to manipulate physiological processes. Because of the
66 safety, availability, and cost-effectiveness of 2-pentanone, this “odourgenetic” approach has great
67 potential for clinical therapeutics.

68

69 **2-Pentanone induces calcium influx in DOR-expressing cells**

70 First, we describe our scheme for DOR cloning, design and activation by 2-pentanone and the
71 process through which physiological functions are manipulated by this system (Fig. 1a and b). To
72 verify whether 2-pentanone bound to and opened these DOR channels on mammalian cells,
73 OR35a and OR83b were expressed in a nonspecific manner in both *in vitro* and *in vivo* systems
74 using a plasmid with a Ubc promoter and an mCherry reporter (Extended Data Fig. 2a). A
75 GCaMP-expressing plasmid and a DOR-expressing plasmid were co-transfected into Neuro-2a
76 cells (Fig. 1c and d) and HEK293T cells (Extended Data Fig. 2b). Calcium influx imaging
77 experiments indicated that bath application of 2-pentanone elicited robust calcium influx in
78 DOR-expressing Neuro-2a cells (Fig. 1c-f) and HEK293T cells (Extended Data Fig. 2b-f). A
79 patch-clamp experiment using Neuro-2a cells showed that bath application of 2-pentanone
80 induced depolarization of the membrane potential, indicating inward rectification by DORs (Fig.
81 1g and h). These results indicated that DORs modulated intracellular calcium levels by inward

82 rectification and might therefore be useful for manipulating calcium-dependent cellular processes.

83

84 **DOR activation elicits spikes in DOR-expressing neurons**

85 To investigate whether DORs manipulated neuronal activity, lentiviruses (LVs) encoding DORs
86 and adeno-associated viruses (AAVs) encoding GCaMP were injected to the S1 cortex of C57
87 mice. Because the length of the DOR sequence is approximately 3.5 kb, a lentivirus vector was
88 chosen for DOR expression in the present study. Neuronal spikes were recorded under current-
89 clamp conditions in cultured neurons and acute brain slices. The fluorescence response to
90 calcium influx was examined by confocal microscopy in acute brain slices. The results showed
91 that 2-pentanone induced robust neuronal spikes in DOR-expressing neurons in cell culture and
92 acute brain slices (Fig. 2a-d). Bath application of 2-pentanone elicited a continuous fluorescence
93 response in DOR-expressing neurons of acute brain slices (Fig. 2e-n and Supplementary Video
94 1). These results indicated that DORs enabled 2-pentanone-driven manipulation of neuronal
95 activity *in vitro* and *in vivo*.

96

97 **DORs enable fast on/off control of behaviour**

98 For DOR expression under the control of the Cre-loxp system in the nervous system, an hSyn
99 promoter following a loxp-stop-loxp sequence was inserted before the DOR sequence (Extended
100 Data Fig. 2a). To determine whether DORs activated specific neurons, these receptors were
101 expressed in GABAergic neurons in the S1 cortex of VGAT-Cre mice. Calcium influx imaging
102 of brain slices was carried out to assess the fluorescence response of GABAergic neurons to 2-
103 pentanone. The fluorescence response of brain slices showed that 2-pentanone activated the
104 target DOR-expressing GABAergic neurons (Fig. 3a-c and Supplementary Video 4).

105 Next, to determine whether 2-pentanone was delivered into the blood by inhalation and crossed
106 the blood–brain barrier into the cerebrospinal fluid (CSF), we examined the 2-pentanone
107 concentrations in the blood and CSF of mice exposed to this compound (2%, v/v) by LC–MS.
108 Plasma containing 2-pentanone and pure plasma were used as positive and negative controls,
109 respectively (Extended Data Fig. 1a-c). 2-Pentanone was detectable in both blood and CSF after
110 a short period of inhalation, indicating that this odorant was transported into the blood and then
111 to the CSF with this simple administration method (Fig. 3d, e and Extended Data Fig. 1d). Time-
112 course examinations were carried out to explore the dynamic profile of the 2-pentanone
113 concentration in the blood of mice and rats exposed to this compound. The 2-pentanone
114 concentration in the blood of both rats and mice showed a time-dependent increase during
115 inhalation of 2-pentanone and decreased rapidly after withdrawal of the odorant (Fig. 3f and
116 Extended Data Fig. 1e). These results indicated that 2-pentanone had the appropriate profile to be
117 a candidate manipulator of this DOR system.

118 The central nucleus of the amygdala (CeA) is a modular command system that exerts
119 integrated control of predatory hunting in mice¹⁷, as we confirmed through an optogenetic
120 experiment in the present study (Supplementary Video 5) to ascertain whether DORs can control
121 predatory hunting behaviours by manipulating CeA neuronal activity. DORs were Cre-
122 dependently expressed in GABAergic neurons in the CeA, and predatory-like bites induced by
123 inhalation of 2-pentanone were observed. The behavioural experiment confirmed that 2-
124 pentanone controlled rodent behaviours by exogenously expressing DORs in target neurons in a
125 non-invasive, fast on/off manner (Fig. 3g-i and Supplementary Video 6).

126

127 **DORs enable fast on/off manipulation of physiological processes**

128 To verify whether DORs reversibly manipulated physiological processes in mice, a lentiviral
129 expressing DORs was injected into the pancreas, skeletal muscle and S1 cortex, respectively. 2-
130 Pentanone was administered to the mice by inhalation; their blood insulin content was then
131 examined using an ELISA, and their blood glucose was examined with a blood glucose meter.
132 Furthermore, the contraction of virus-injected muscles activated by 2-pentanone was observed
133 under a stereomicroscope, along with the associated limb movement. Neuronal activity was
134 examined by *in vivo* calcium influx imaging. A few minutes of 2-pentanone inhalation resulted in
135 a significant increase in the blood insulin concentration in pancreatic DOR-expressing mice and
136 therefore lowered the blood glucose level (Fig. 4a-c). Inhalation of 2-pentanone elicited muscle
137 contraction and limb movements within a few minutes, and the effect persisted until the odorant
138 was withdrawn (Fig. 4d-f). Calcium influx imaging in the S1 cortex *in vivo* showed that 2-
139 pentanone inhalation evoked robust continuous neuronal firing within a few minutes, which
140 persisted until 2-pentanone withdrawal (Extended Data Fig. 3a-d and Supplementary Video 2).
141 Repeated inhalation of 2-pentanone elicited another bout of neuronal firing. These results
142 confirmed that DORs enabled reversible manipulation of several physiological processes *in vivo*.

143 In the present study, DORs overcome many limitations of other methods, including the need
144 for expensive specialized equipment; the difficulty of delivering light to widely distributed cell
145 populations; the invasive procedures required to activate optogenetic systems in deep tissue; and
146 the long, slow pharmacodynamics and irreversible metabolic processes of the designer drugs
147 used in chemogenetics^{6,9}. 2-Pentanone is a naturally produced phytochemical that is present in
148 bananas¹⁸ and carrots¹⁹; this colourless liquid ketone has an acetone-like or intensely fruity odour.
149 It is sometimes used in very small amounts as a food additive to impart flavour. 2-Pentanone is
150 soluble in water and volatilizes rapidly to a gas at room temperature. Therefore, it was very easy

151 to administer by inhalation to manipulate our DOR system. Furthermore, the compound is
152 eliminated very quickly, mainly via exhalation, without a significant metabolic process. This
153 profile indicates the good controllability of systemic 2-pentanone levels in the present DOR
154 system, providing an easy-to-use tool that has potential for clinical applications in the treatment
155 of various diseases, such as diabetes, Parkinson's disease, and neocortical seizures²⁰.
156

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202 **Figure legends**

203 **Fig. 1. *Drosophila*-derived DORs control cellular activity**

204 (a, b) Schematic drawing showing the principle of DOR design and their activation by 2-
205 pentanone to manipulate physiological functions. (c, d) Two frames (before and after application
206 of 2-pentanone) of time-lapse calcium influx images of GCaMP co-expressed with DORs or a
207 control in Neuro-2a cells. The fluorescence responses showed that 10 μ M 2-pentanone evoked
208 significant calcium influx in DOR-expressing cells. (e, f) Time course of fluorescence responses
209 from regions of interest (ROIs), showing a robust calcium influx in DOR-expressing cells in
210 response to 10 μ M 2-pentanone. (g, h) Changes in the membrane potential change of current-
211 clamped Neuro-2a cells; bath application of 10 μ M 2-pentanone significantly depolarized the
212 membrane potential of DOR-expressing cells (mean change = 16.38 ± 7.79 mV, n=25 cells).

213 **Fig. 2. DORs enable 2-pentanone-driven neuronal spiking**

214 (a, c) Voltage traces showing spikes in single current-clamped neurons of cultured cortical
215 neurons and brain slices. Compared with the control, bath application of 10 μ M 2-pentanone
216 evoked robust firing spikes of DOR-expressing neurons in brain slices and cultured cortical
217 neurons. (b, d) Spikes in current-clamped neurons in brain slices and cultured cortical neurons in
218 response to application of 2-pentanone (slice: Control, 1.1 ± 0.6 Hz, DORs, 6.9 ± 3.1 Hz, p=0.000,
219 n=36 neurons from nine mice; cultured neurons: Control, 0.8 ± 0.5 Hz, DORs, 6.1 ± 3.6 Hz,
220 p=0.000, n=34 neurons from six cultures, non-parametric Mann–Whitney rank-sum test, two-
221 sided). Experiments were repeated independently more than five times with similar results.
222 (e, i) Two frames (before and after application of 2-pentanone) of time-lapse calcium influx
223 images of brain slices from C57 mice co-infected with AAV-GCaMP and LV-DOR or LV-control.
224 Compared with the control, 10 μ M 2-pentanone evoked significant calcium influx in DOR-

225 expressing neurons. **(f, j)** Snapshots of fluorescence responses of control or DOR-expressing
226 sample neurons (marked with white rectangles on left images) to 2-pentanone. **(g, k)** Time course
227 of fluorescence responses to 2-pentanone in the above neurons. **(h, l)** Mean fluorescence
228 intensity change ($\Delta F/F_0$) of DOR-expressing and control neurons in response to 2-pentanone
229 (DORs: $1.56\% \pm 0.18\%$; control: $0.18\% \pm 0.02\%$, $p=0.001$, $n = 25$ neurons from eight mice). **(m,**
230 **n)** Time course of fluorescence responses of control and DOR-expressing neurons in ROIs
231 during application of 2-pentanone. Compared with the control, DOR-expressing neurons showed
232 enhanced fluorescence responses to $10 \mu\text{M}$ 2-pentanone.

233 **Fig. 3. DORs activate GABAergic neurons in the CeA and control predatory-like**
234 **behaviours**

235 **(a)** Time-lapse calcium influx images of brain slices from VGAT-Cre mice co-infected with AAV-
236 GCaMP and LV-hSyn-LSL-DORs or a control virus; bath application of $10 \mu\text{M}$ 2-pentanone
237 evoked significant calcium influx in DOR-expressing neurons. **(b, c)** Time course of
238 fluorescence responses of control and DOR-expressing neurons in regions of interest (ROIs)
239 during application of 2-pentanone. Compared with the control, DOR-expressing neurons showed
240 enhanced fluorescence responses to 2-pentanone. **(d, e)** The concentration of 2-pentanone in the
241 blood of mice was examined by LC-MS after inhalation of 2-pentanone (2%, v/v) for 3 min. 2-
242 Pentanone was identified by the retention time (approximately 2.74 min) and the mass charge
243 ratio ($m/z = 87$). **(f)** Dynamic concentration of 2-pentanone in the blood of rats exposed to this
244 compound. The 2-pentanone concentration showed a rapid time-dependent increase within a few
245 minutes after inhalation, and the concentrations remained elevated until withdrawal of the
246 stimulus. Upon withdrawal of 2-pentanone, concentration decreased to approximately 20% of the
247 peak concentration within 10 minutes ($48.88 \pm 10.63 \text{ ng/ml}$ to $12.11 \pm 3.76 \text{ ng/ml}$, $n=4$ for each

248 time point). (g) Schematic of virus injection into the CeA and inhalation of 2-pentanone in freely
249 moving mice. (h, i) Values reflecting predatory-like behaviours evoked by 2-pentanone.
250 Compared with the control, both the number of bites and the total time spent biting were
251 increased in DOR-expressing mice (n= 6, non-parametric Mann–Whitney rank-sum test, two-
252 sided).

253 **Fig. 4. DORs manipulate physiological functions *in vivo***

254 (a) Schematic of virus injection into the pancreas and inhalation of 2-pentanone in freely moving
255 mice. (b) Concentrations of insulin in the blood of mice after inhalation of 2-pentanone (control:
256 4.82 ± 1.04 mIU/L; DORs: 9.32 ± 3.34 mIU/L, $p=0.001$, $n=27$ mice, t test, two-sided) are shown. (c)
257 Time course of the blood glucose change in mice subjected to 2-pentanone inhalation. Compared
258 with the control, blood glucose began to decrease within a few minutes of 2-pentanone inhalation,
259 and this change persisted for more than 10 minutes after withdrawal of 2-pentanone. Data are
260 shown as the mean \pm s.e.m. ($n = 25$, $*p<0.05$, repeated-measures ANOVA). (d) Schematic of
261 virus injection into muscles and inhalation of 2-pentanone using a mask. (e) The image shows a
262 virus-injected muscle marked by a green circle. (f) Vertical blue lines represent the muscle
263 contraction evoked by 2-pentanone. 2-Pentanone elicited continuous contraction of muscles
264 expressing DORs.

265

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268

269 **Author contributions**

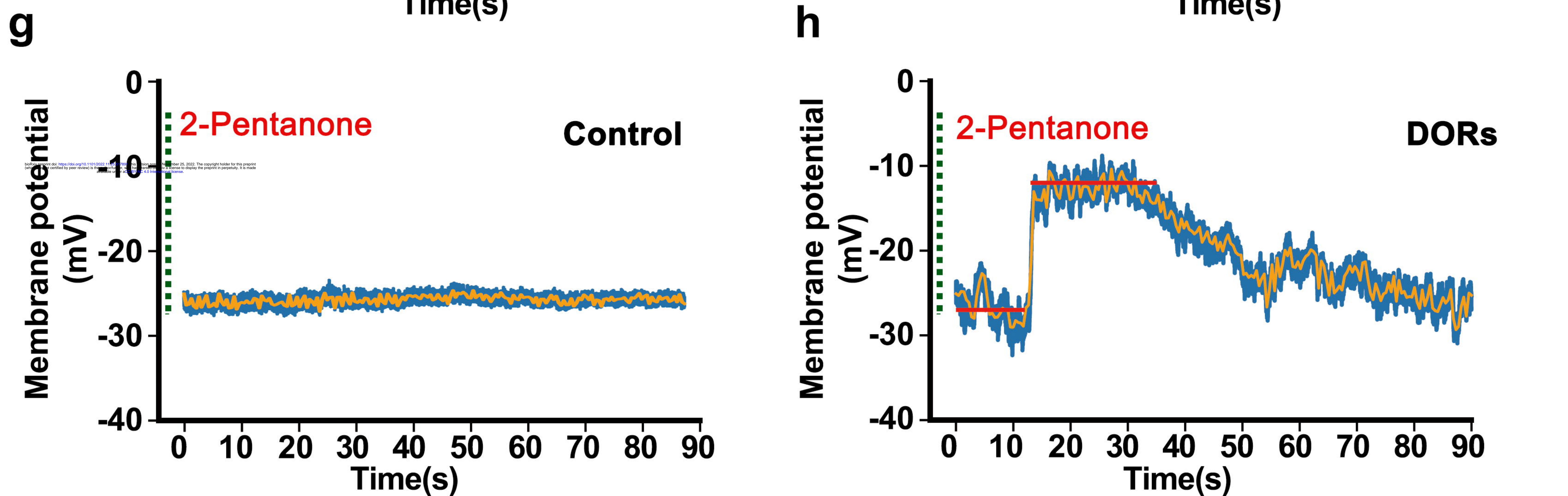
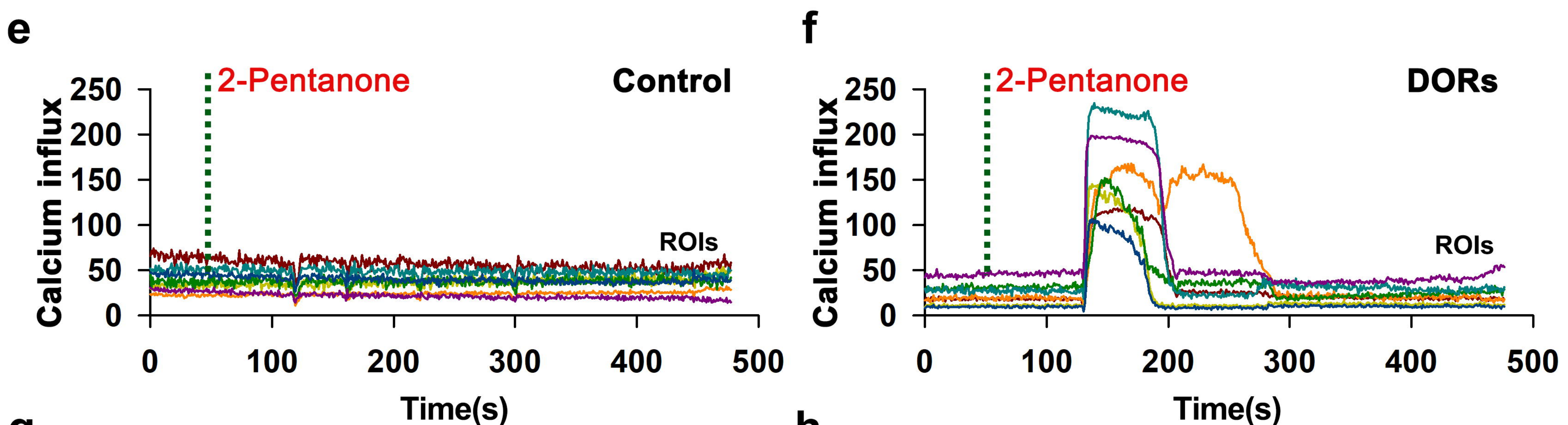
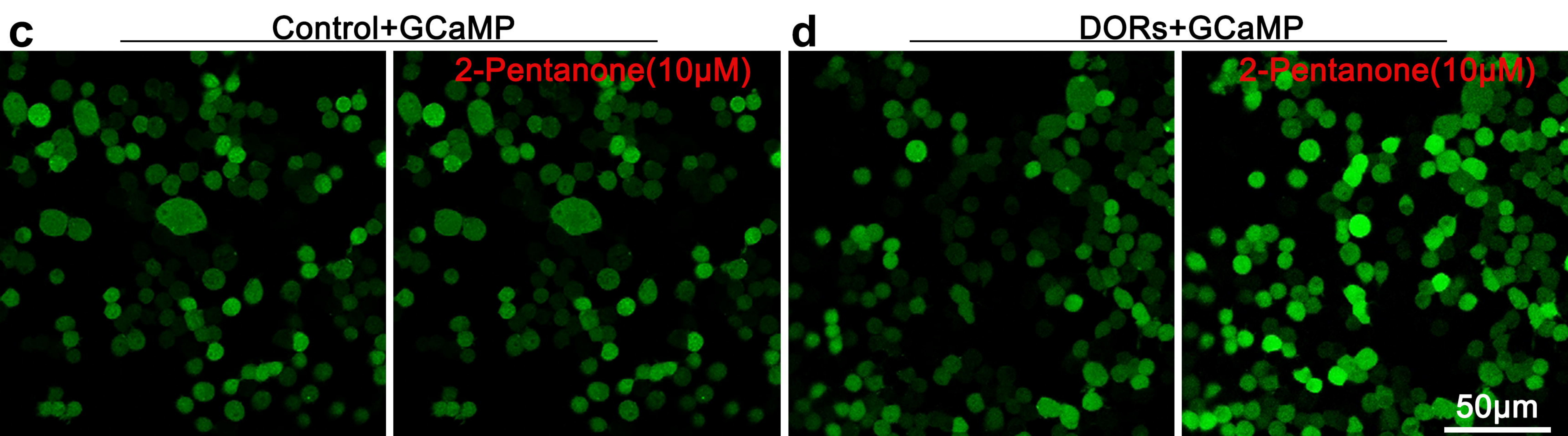
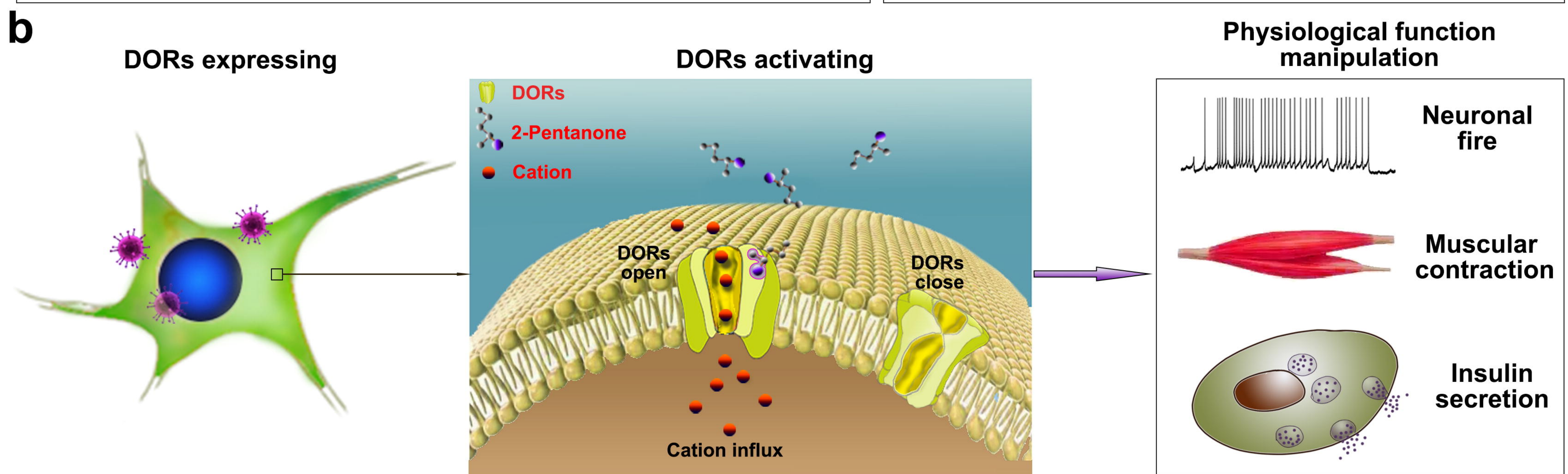
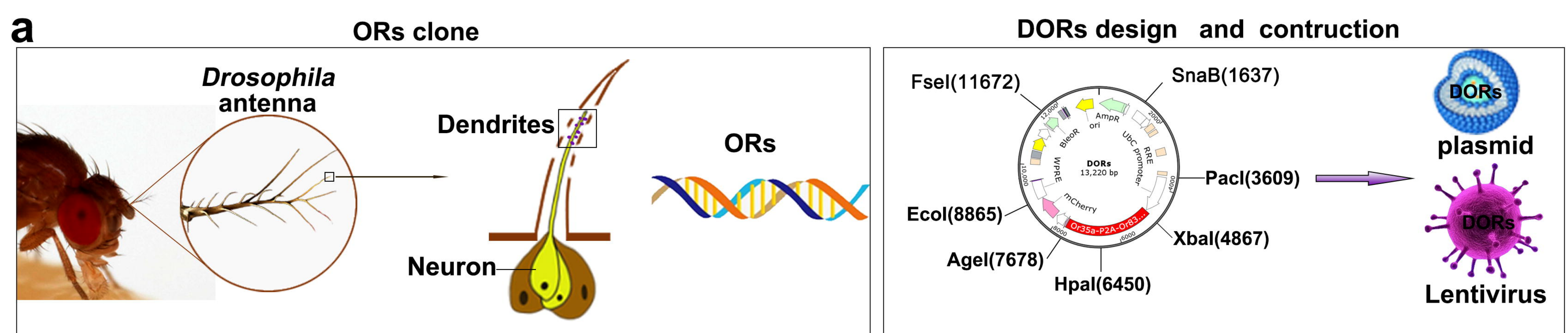
270 S.C.S carried out electrophysiological and viral injection; Y.Q.W designed and carried out
271 behaviour test, analyzed data and wrote the paper; X.Q.X carried out plasmid and viral
272 construction and cellular expressing; X.C.H designed and carried out 2-pentanone examination;
273 W.L carried out 2-pentanone examination; X.H.L carried out calcium imaging; L.H.W carried out
274 blood insulin and glucose test; W.T carried out muscle contraction experiments. Y.G carried out
275 cells and neuron culture; G.C designed the study and analyzed data; C.B.K designed the study,
276 analyzed data and wrote the paper.

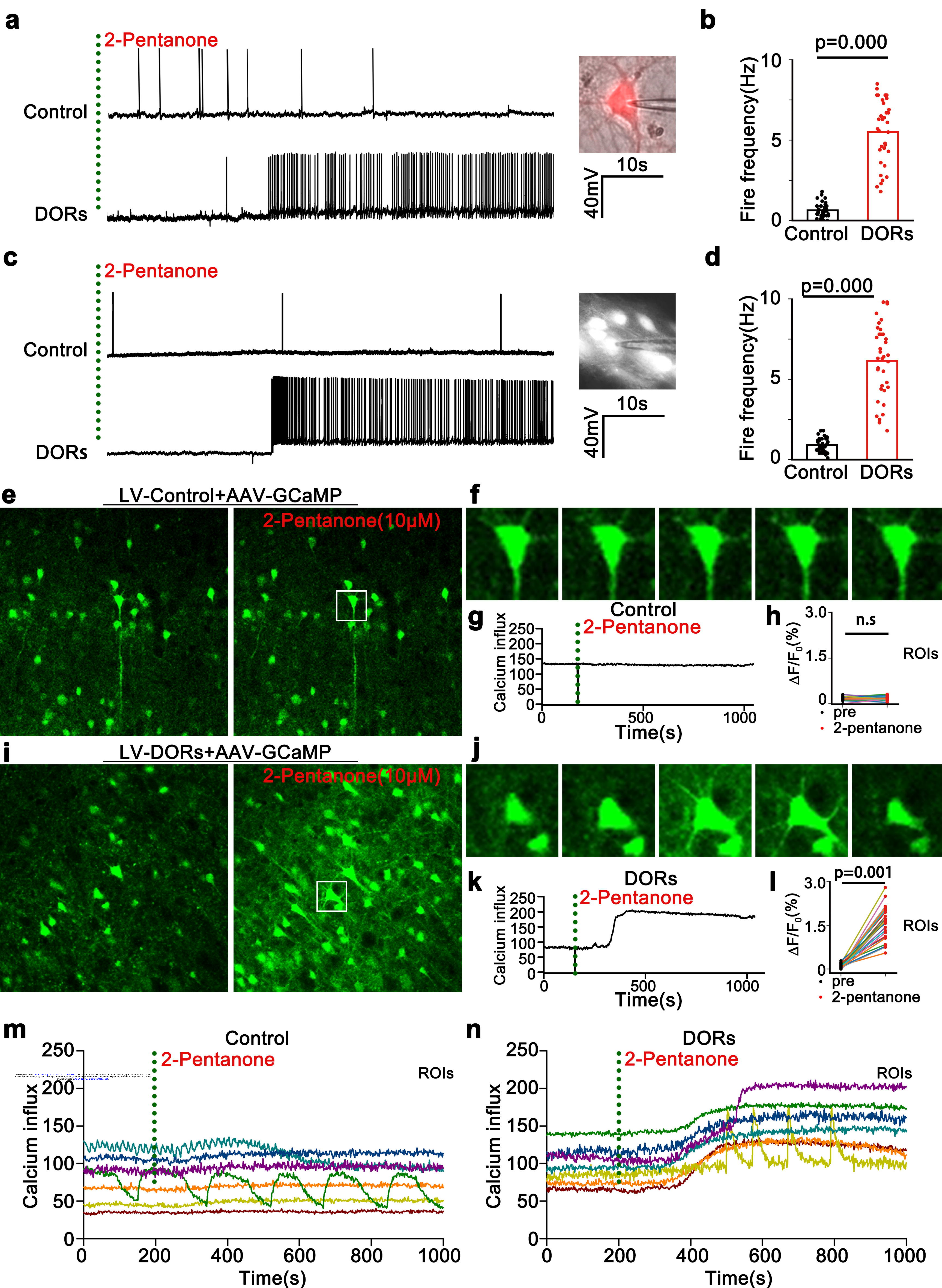
277 **Competing interests**

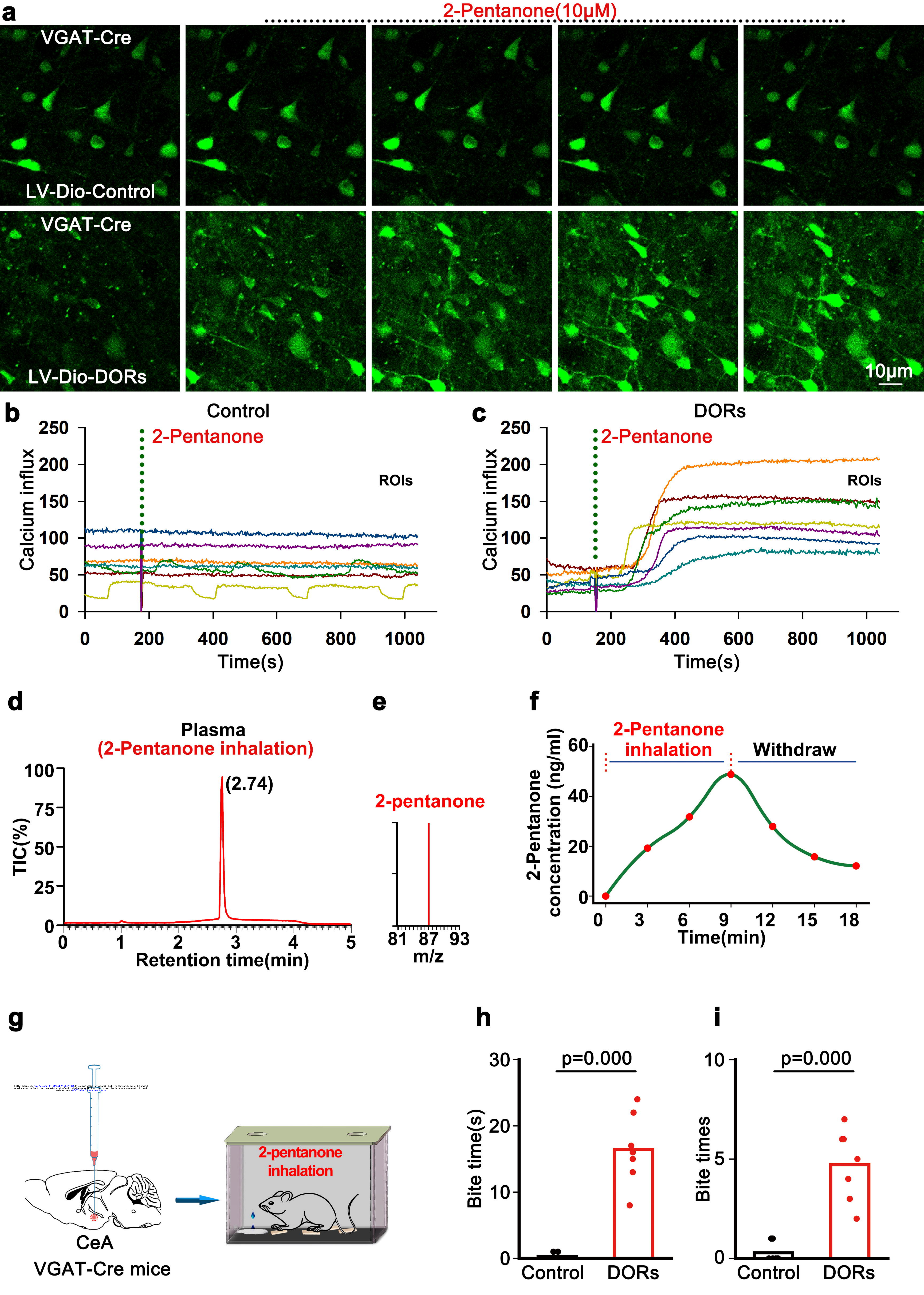
278 Changbin Ke has filed patent applications whose value might be affected by this publication.

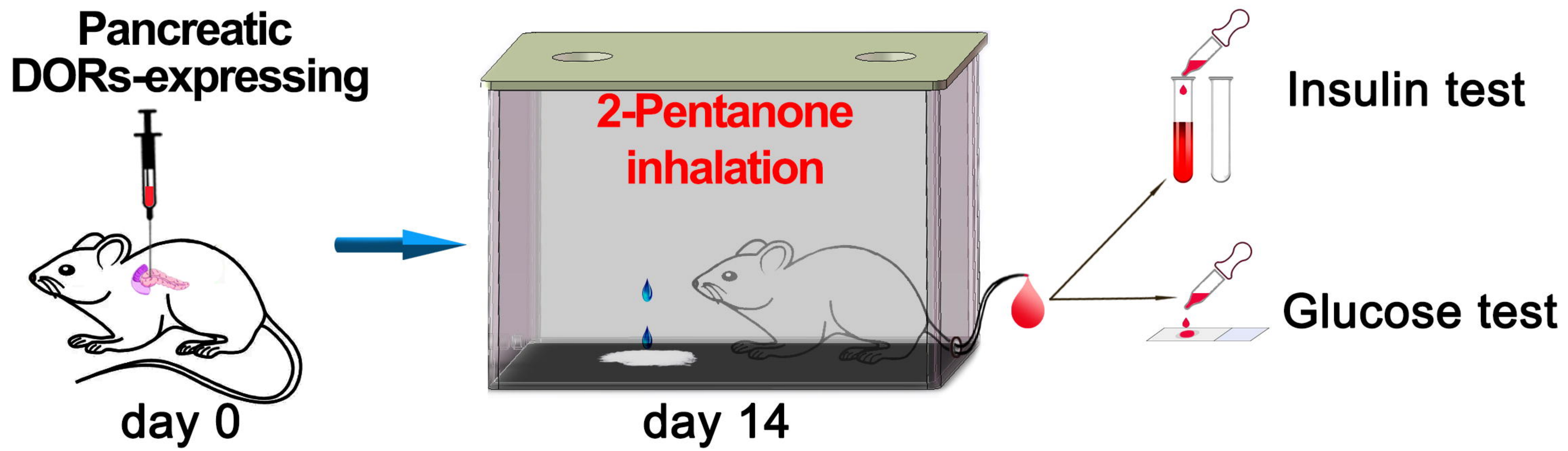
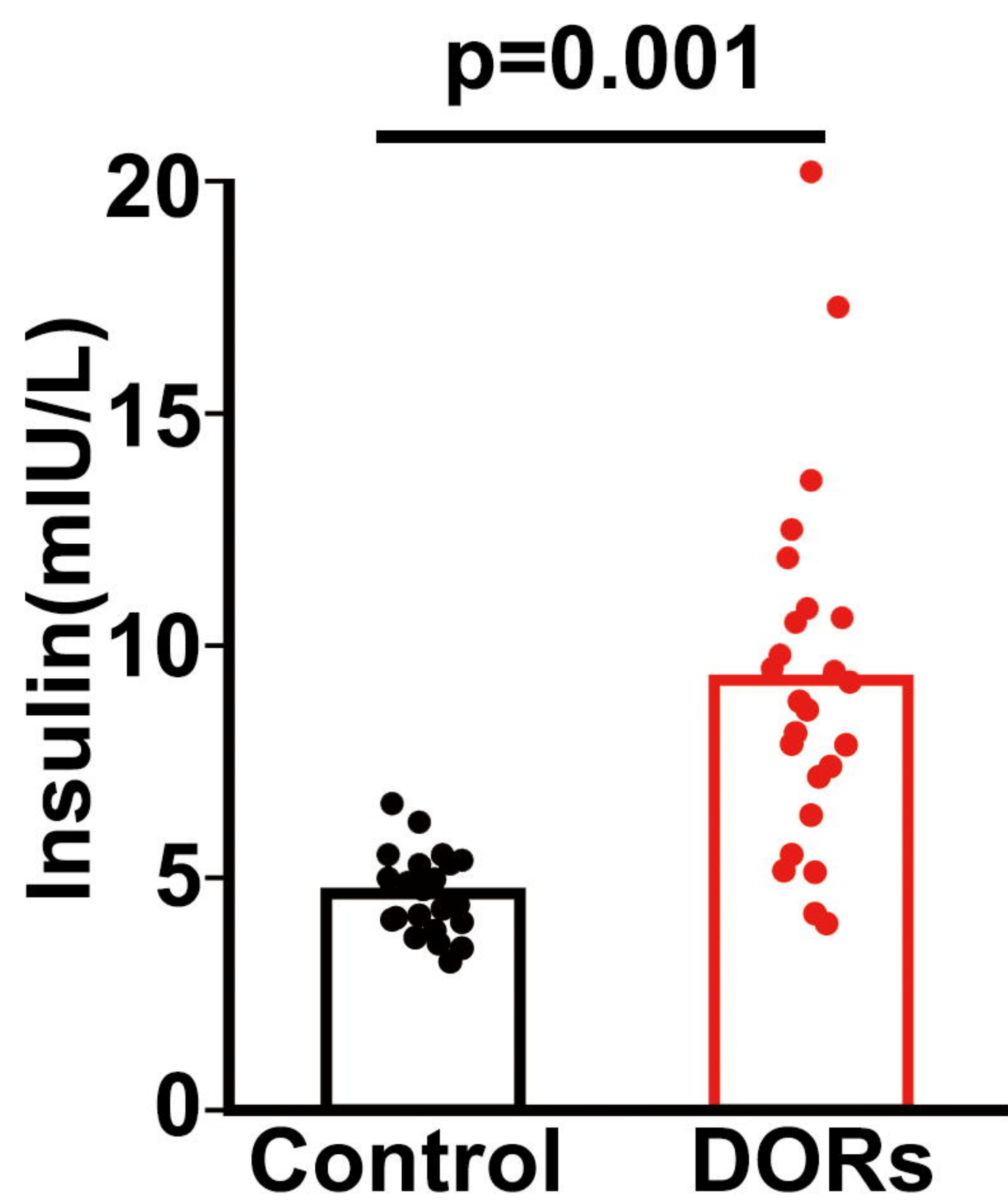
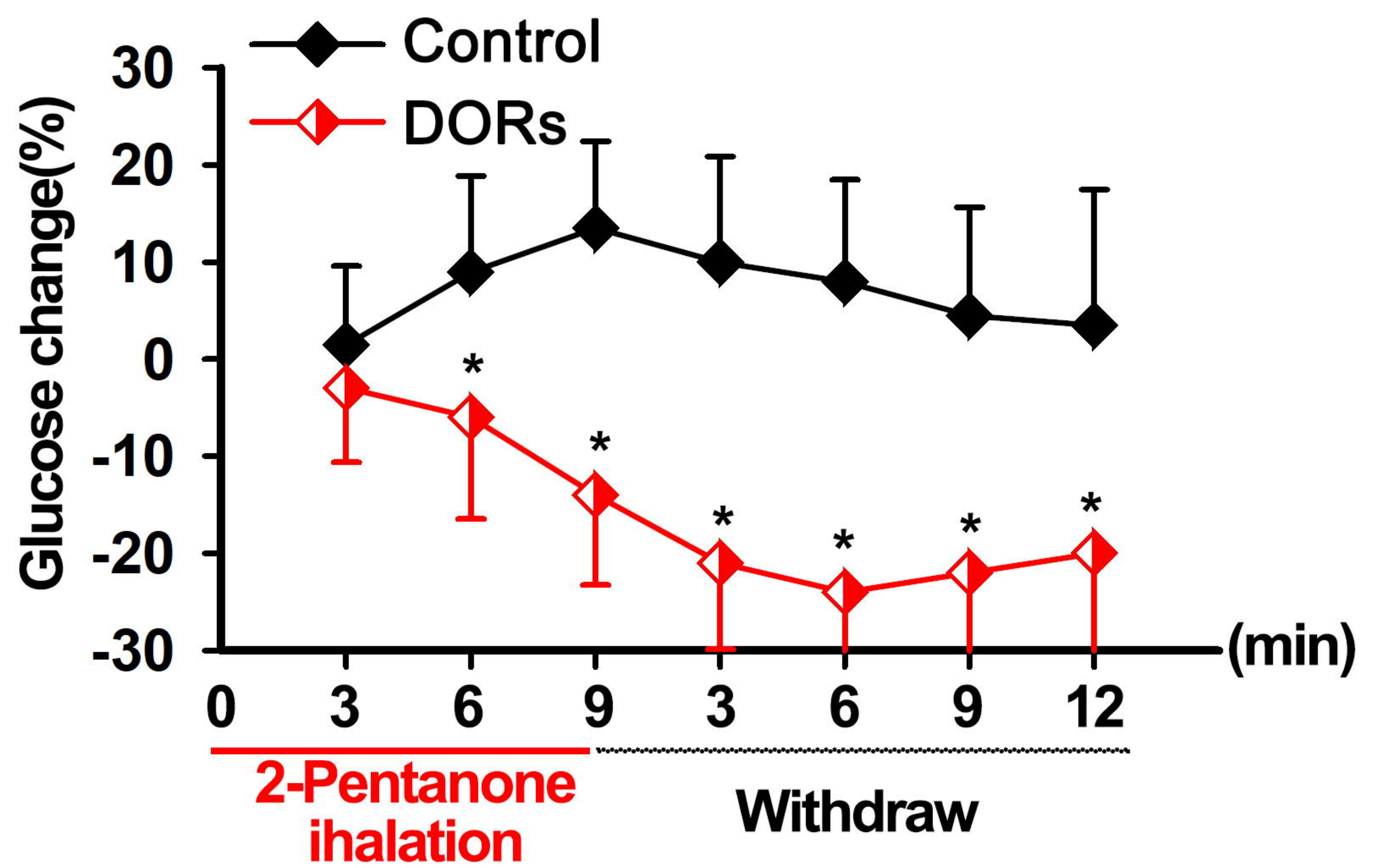
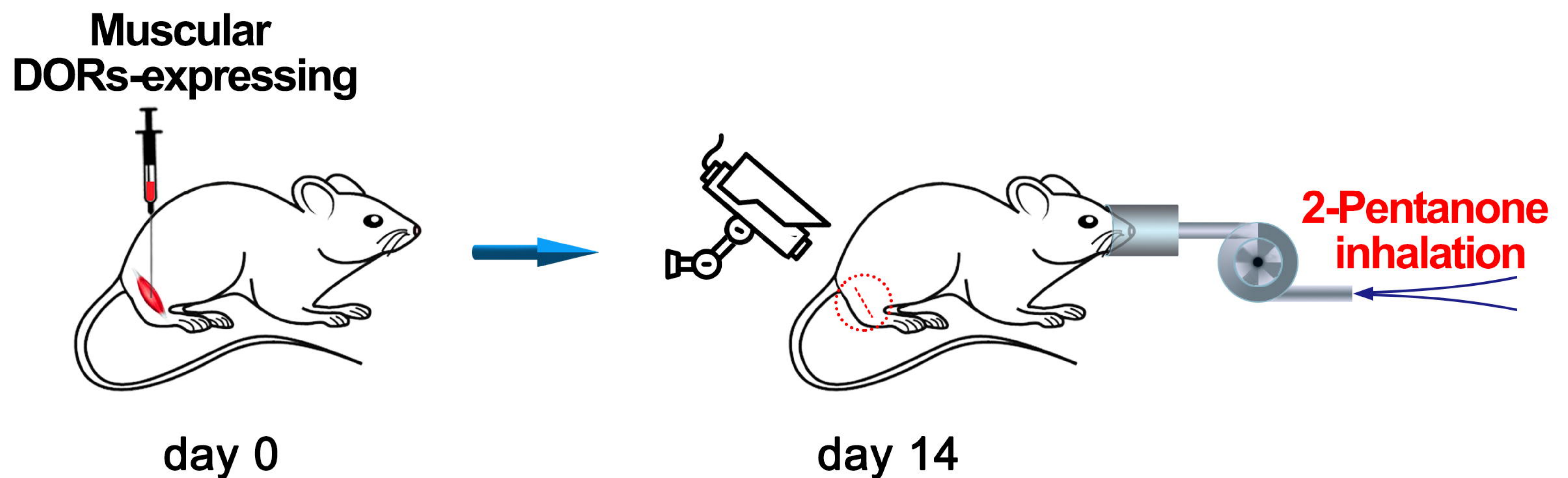
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