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SHORT COMMUNICATION

Paired pulse ratio analysis of insulin-induced synaptic plasticity in the snail brain

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SUMMARY

Insulin's action in the brain can directly alter cognitive functioning. We have recently shown that molluscan insulin-related peptides are upregulated following a conditioned taste aversion (CTA) training procedure. In addition, when mammalian insulin is superfused over the isolated *Lymnaea stagnalis* central nervous system, it elicits long-term synaptic enhancement at the monosynaptic connection between the cerebral giant cell and the buccal 1 (B1) motor neuron. This synaptic enhancement is thought to be a neural correlate of CTA. Here, we examined whether the observed changes in synaptic plasticity were the result of presynaptic and/or postsynaptic alterations using the paired pulse procedure. The paired pulse ratio was unaltered following insulin application, suggesting that insulin's effects on synaptic plasticity are mediated postsynaptically in the B1 motor neuron. Thus, it was suggested that postsynaptic changes need to be considered when insulin's actions on synaptic plasticity are examined.

Key words: conditioned taste aversion, insulin, *Lymnaea*, paired pulse ratio, synaptic plasticity.

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INTRODUCTION

Insulin is thought to play an important role in cognitive functioning in animals, including humans (Fernandez and Torres-Alemán, 2012). Insulin receptors are found in abundance on neurons in the hippocampus, the entorhinal cortex and the frontal cortex, all areas important for memory formation and retrieval (Chiu et al., 2008). However, little is known at the neurophysiological level as to how insulin alters memory.

The pond snail *Lymnaea stagnalis* is widely used as a model system to elucidate the cellular and molecular mechanisms that underlie learning and memory (Benjamin, 2012). Our group has primarily focused our attention on conditioned taste aversion (CTA) in this model system (Kojima et al., 1996). We previously found that molluscan insulin-related peptides (MIPs) were upregulated in snails exhibiting CTA (Azami et al., 2006). More recently, we have shown at the electrophysiological level that application of mammalian insulin or MIPs to the isolated central nervous system (CNS) evoked long-term synaptic enhancement at the serotonergic excitatory monosynaptic connection between the cerebral giant cell (CGC) and the buccal 1 (B1) motor neuron (Murakami et al., 2013). That is, the excitatory postsynaptic potentials (EPSPs) recorded in the B1 motor neuron elicited by activation of the CGC were significantly increased when insulin was applied to the CNS. This change in synaptic efficacy was hypothesized to be the neural correlate of behavioral CTA (Kojima et al., 1996; Murakami et al., 2013).

Here we examined whether insulin's effects at this excitatory monosynaptic connection are mediated at the presynaptic and/or postsynaptic neuron. To accomplish this, we made use of the paired pulse procedure. In this procedure, we compared the amplitude of an initial EPSP with that of a second EPSP elicited shortly after the first EPSP. If the second EPSP is larger than that of the first EPSP, it is called paired pulse facilitation, whereas if the second EPSP is smaller, it is called paired pulse depression. The assumption made in paired pulse facilitation is that there is a residual 'calcium cloud' present from the first action potential such that the second action potential will now cause more transmitter to be released, because there is more calcium around (Schulz et al., 1994). Thus, paired pulse facilitation is considered to be a presynaptic phenomenon. However, if insulin application caused a decrease in the paired pulse ratio, we would label this result as paired pulse depression. That is, insulin application would again alter the amount of transmitter release from the presynaptic neuron, albeit a decrease in the amount released by the second action potential. Here, we report that the paired pulse ratio is unaltered following insulin application, suggesting that insulin's effects on synaptic plasticity are mediated postsynaptically in the B1 motor neuron.

MATERIALS AND METHODS

Snails

Specimens of *Lymnaea stagnalis* (L.) with a 15–25 mm shell (young adults) were obtained from our snail-rearing facility. All snails were maintained in dechlorinated tapwater (i.e. pond water) under a 12 h:12 h light:dark cycle at 20°C and fed *ad libitum* on a kind of turnip leaf, *Brassica rapa* var. *peruviridis* [komatsuna (in Japanese)] and spiral shell food flakes (Nisso, Saitama, Japan) every other day. Snails were anesthetized with 25% Listerine® before dissection.

Electrophysiology

We examined the properties of the chemical synapses between the CGC and its ipsilateral B1 motor neuron in the isolated *L. stagnalis*

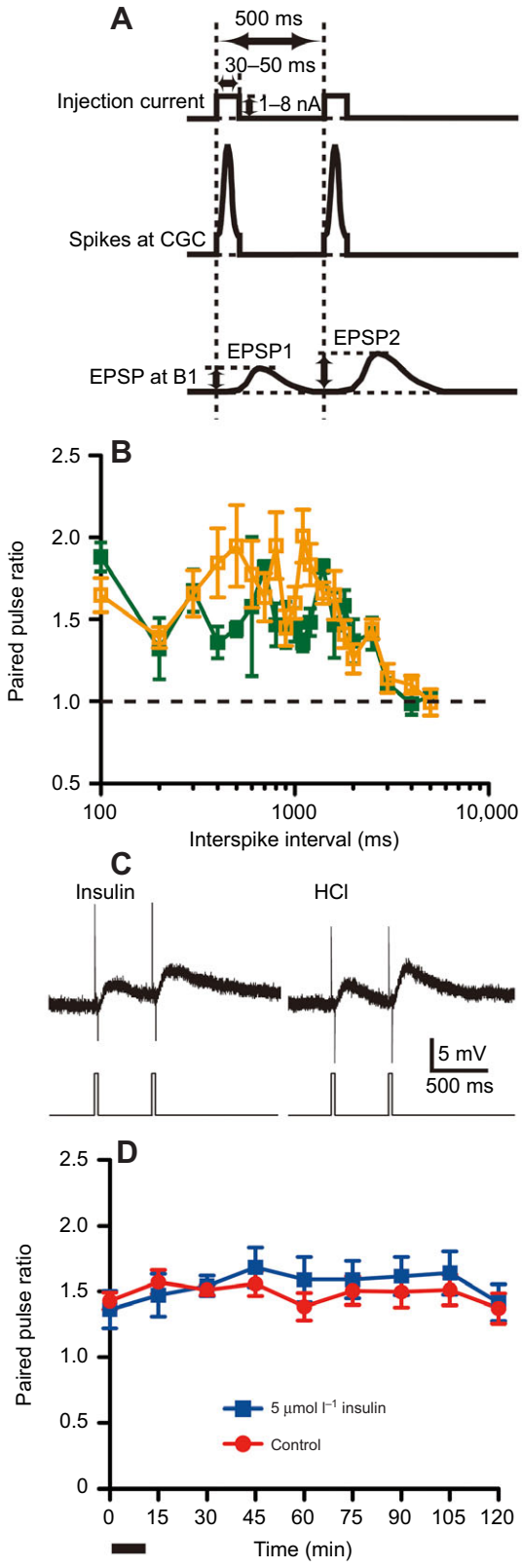

Fig. 1. Paired pulse ratio between two single excitatory postsynaptic potentials (EPSPs) recorded in the buccal 1 (B1) motor neuron of *Lymnaea stagnalis*. (A) Illustration of timing for paired pulse ratio. We injected depolarizing current in the cerebral giant cell (CGC) presynaptic neuron twice and evoked two single spikes. We simultaneously recorded two single EPSPs (EPSP1 and EPSP2) in the B1 motor neuron. The paired pulse ratio was obtained as the peak amplitude of EPSP2 divided by that of EPSP1. (B) Relationship between paired pulse ratio and inter-pulse interval. The paired pulse facilitation (i.e. a paired pulse ratio >1) was observed at inter-pulse intervals between 100 and 3000 ms. Two examples are presented here. Orange data points are means ± s.e.m. of three snails, and green data points are means ± s.e.m. of five snails. The *x*-axis is expressed as a log scale. (C) Typical data for a first EPSP and a following facilitated postsynaptic potential. Left: 5 µmol l^{-1} bovine insulin was applied for 10 min and washed out, and then the paired pulse experiments were performed with an inter-pulse interval of 500 ms. Right: 14 µmol l^{-1} HCl saline control. (D) No change in the paired pulse ratio after the superfusion of 5 µmol l^{-1} bovine insulin. There were no significant differences between the data for bovine insulin and those for control (14 µmol l^{-1} HCl), suggesting that long-term synaptic enhancement induced by insulin is mainly due to changes in the B1 postsynaptic neuron. The duration of the pulse was 30–50 ms. We obtained the data twice at each time point from nine preparations each for bovine insulin and control.

CNS. The paired CGCs play a key role in the control of the feeding system in *L. stagnalis*. The B1 motor neuron, which innervates the salivary gland, receives a monosynaptic excitatory input from the CGC, and is activated during the radula-protraction phase of the feeding cycle (Benjamin, 2012). The CGC-B1 synapse is a good model with which to elucidate how changes in synaptic transmission serve as a neural correlate of CTA behavioral memory.

The CNSs were dissected from snails in ice-cold *L. stagnalis* saline. The *L. stagnalis* saline consisted of 50 mmol l^{-1} NaCl, 1.6 mmol l^{-1} KCl, 2.0 mmol l^{-1} $MgCl_2$, 3.5 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} HEPES (pH 7.9). For the paired pulse procedure, we stimulated the CGC with two single pulses (injection current: 1–8 nA, duration: 30–50 ms, inter-pulse interval: 500 ms or other specified values; electric stimulator: SEN-7203, Nihon Kohden, Tokyo, Japan) by a current clamp method and recorded two single EPSPs in the B1 motor neuron (intracellular recording amplifier: MEZ-8300, Nihon Kohden; AD converter: DIGIDATA 1322A, Axon Instruments, Foster City, CA, USA). The depolarizing current and the duration of the pulse were adjusted so as to result in a single spike in the CGC. We subtracted the tail of the first EPSP from the second one and obtained the peak amplitudes of the first EPSP and the second one. In these experiments, 5 µmol l^{-1} bovine insulin (Sigma-Aldrich, St Louis, MO, USA) was applied for 10 min, and then it was washed out with the saline. The stock solution of bovine insulin was prepared as 2 mg ml^{-1} (i.e. 349 µmol l^{-1}) in 1 mmol l^{-1} HCl, and thus the final concentration of HCl included in the bovine insulin solution was 14 µmol l^{-1}. We therefore used 14 µmol l^{-1} HCl dissolved in saline for the control experiments. Because previous studies using another mollusc, *Aplysia*, demonstrated that application of 5 µmol l^{-1} bovine insulin activated the bag cell-neuron insulin receptor by stimulating its autophosphorylation on tyrosine residues and evoked the egg-laying hormone secretion (Jonas et al., 1997), bovine insulin was also used in our experiments instead of MIPs.

Statistics

The data are expressed as means ± s.e.m. Significant differences between two groups were examined by two-way repeated-measures ANOVA ($P<0.05$).

RESULTS AND DISCUSSION

Contribution from a presynaptic neuron and/or a postsynaptic neuron to a long-term synaptic enhancement

We used the paired pulse procedure (Fig. 1A) to investigate the extent to which insulin-induced synaptic enhancement of the CGC-B1

synapse was the result of a change in the presynaptic (the CGC) and/or the postsynaptic neuron (the B1 motor neuron). We first determined an appropriate inter-pulse interval that would produce a facilitated postsynaptic potential. As can be seen in Fig. 1B, a facilitated postsynaptic potential was observed when the inter-pulse interval was under 3 s. We thus decided on a 500 ms inter-pulse interval for the paired pulse ratio experiments. This inter-pulse interval was not particularly long, given that the duration of a single spike in *L. stagnalis* neurons was approximately 25 ms.

We applied 5 μmol l^{-1} bovine insulin to the isolated CNS for 10 min, washed out the solution, and then stimulated the CGC with two single pulses with an inter-pulse interval of 500 ms. As expected, we found that the peak amplitude of the second EPSP was larger than that of the first (Fig. 1C, left panel). Similar data were obtained with the 14 μmol l^{-1} HCl saline control (Fig. 1C, right panel). When insulin was applied and then washed out, we found that during the 2 h recording period, the paired pulse ratio was unaltered (P>0.05; Fig. 1D). These data are consistent with the hypothesis that the long-term synaptic enhancement observed at the CGC-B1 synapses was the result of a change in properties of the B1 postsynaptic neuron rather than a change in the CGC presynaptic neuron.

The serotonergic EPSP in the B1 motor neuron is mediated *via* at least two types of serotonin receptors: metabotropic 5-HT_2-like receptors and ionotropic 5-HT_3-like receptors (Kawai et al., 2011). The fast component of compound EPSP is mediated by 5-HT_3-like receptors, and the slow component is generated *via* 5-HT_2-like receptors. Because 5-HT_3-like receptors are ionotropic, the analysis of paired pulse ratio is thought to be reasonable even when we used a long inter-pulse interval, such as 500 ms.

Possible function of insulin in a long-term synaptic enhancement

Using cultured mammalian neurons that respond to γ-aminobutyric acid (GABA), application of insulin increased the expression of the $GABA_A$ receptors on the postsynaptic membranes of these neurons (Wan et al., 1997). Insulin may evoke a rapid recruitment of functional $GABA_A$ receptors to the postsynaptic membrane, suggesting a fundamental mechanism for the generation of synaptic plasticity. At the CGC-B1 synapses in *L. stagnalis*, serotonin appears to be the likely transmitter substance (Kawai et al., 2011), so it is possible that MIPs could result in an increase in serotonin receptors (possibly ionotropic 5-HT_3-like receptors) on the B1 motor neurons. This possibility is currently being examined.

In conclusion, the effects of exogenous insulin on synaptic enhancement of a synapse that is thought to be a neural correlate of CTA probably occur in the postsynaptic neuron. That is, the buccal motor neurons are altered by insulin in the CTA of *L. stagnalis*. Our findings here in *L. stagnalis* further emphasize the fact that in invertebrate model systems [e.g. *Aplysia* (see Glanzman, 2010)], changes occurring postsynaptically must be considered when the neuronal basis of memory formation is studied.

AUTHOR CONTRIBUTIONS

J.M., R.O., Y.F., M.S., K.L. and E.I. conceived and designed the experiments. J.M. and R.O. performed the experiments. J.M., R.O., Y.F., M.S., K.L. and E.I. drafted and revised the article.

COMPETING INTERESTS

No competing interests declared.

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