BRIEF REPORT / RAPPORT BREF

Insulin-like peptides are not involved in maturation or functional recovery of neural circuits in the locust flight system

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Abstract: We sought to manipulate maturation and functional recovery of locust flight circuitry by treating locusts with pharmacological doses of bovine anti-insulin and insulin. Anti-insulin treatment of maturing locusts caused reduced growth of the thoracic nervous system, lower body weight, and softer cuticles compared with control locusts. We were unable to block either maturation or recovery of flight circuitry with anti-insulin. We propose that insulin-related peptides are involved in growth and cuticular changes during adult maturation, but have no role in promoting neuronal sprouting during this period or as a result of injury.

Key words: insulin, maturation, functional recovery, proprioceptors, flight.

Résumé : L'insuline joue un rôle de facteur de différenciation et de croissance dans le système nerveux. Nous avons eu pour objectif de manipuler la maturation et le rétablissement fonctionnel des circuits neuronaux du système de vol des locustes en traitant ces derniers avec de l'anti-insuline et de l'insuline. Le traitement anti-insulinique a réduit la croissance du système nerveux thoracique, diminué le poids et ramolli les cuticules chez les locustes en développement par comparaison aux locustes témoins. Ce traitement n'a pas permis de bloquer la maturation ou le rétablissement des circuits du système de vol. Nous concluons que les peptides apparentés à l'insuline jouent un rôle dans les variations de croissance et cuticulaires durant la maturation, mais qu'ils n'ont aucune influence sur le bourgeonnement neuronal durant cette période ou à la suite d'une lésion.

Mots clés : insuline, maturation, rétablissement fonctionnel, propriocepteurs, vol.

[Traduit par la Rédaction]

Introduction

Members of the insulin and insulin-like growth factor family of peptides are found in both vertebrates and invertebrates and are important mediators of functional plasticity in nervous tissue (de Pablo and de la Rosa 1995; Torres-Aleman 1999). Insulin stimulates neurite outgrowth (Fernyhough et al. 1993), enhances nerve regeneration (Ekström et al. 1993) and can prevent learning deficits in streptozotocin-diabetic rats (Biessels et al. 1998) possibly via CNS synapse-specific pathways (Abbott et al. 1999). The locust flight system is a well-established model for investigations of neural growth processes affecting motor pattern generation (e.g., Wolf and Büschges 1997). We tested whether insulin-like factors have a role in mediating neural plasticity in this model system.

Invertebrate forms of insulin, including locust insulin-related peptide (LIP), have been implicated as mediators of neuronal differentiation and growth (e.g., de Pablo et al. 1988), as neuromodulators, and possibly as neurotransmitters (e.g., Shapiro et al. 1991). In *Drosophila melanogaster*, insulin receptors have been localized to developing nervous tissue (Garofalo and Rosen 1988) and the neuromuscular junction (Gorczyca et al. 1993). Moreover, activity of the *D. melanogaster* insulin receptor gene is required for normal development of the central nervous system (Fernandez et al. 1995). Significantly, addition of bovine insulin to cultures of locust neurons promotes neurite outgrowth (Vanhems et al. 1990). These findings strongly suggest that LIP could play a role in neuronal growth processes in the locust.

In adult locusts, meso- and metathoracic neurons of the locust flight system grow during maturation (Gee and Robertson 1994) and during recovery from ablation of the hindwing tegulae (Büschges et al. 1992). During adult maturation, there is an increase in wingbeat frequency (WBF) (Kutsch 1973), the thoracic ganglia increase in size, and flight muscle mass increases. WBF decreases following ablation of

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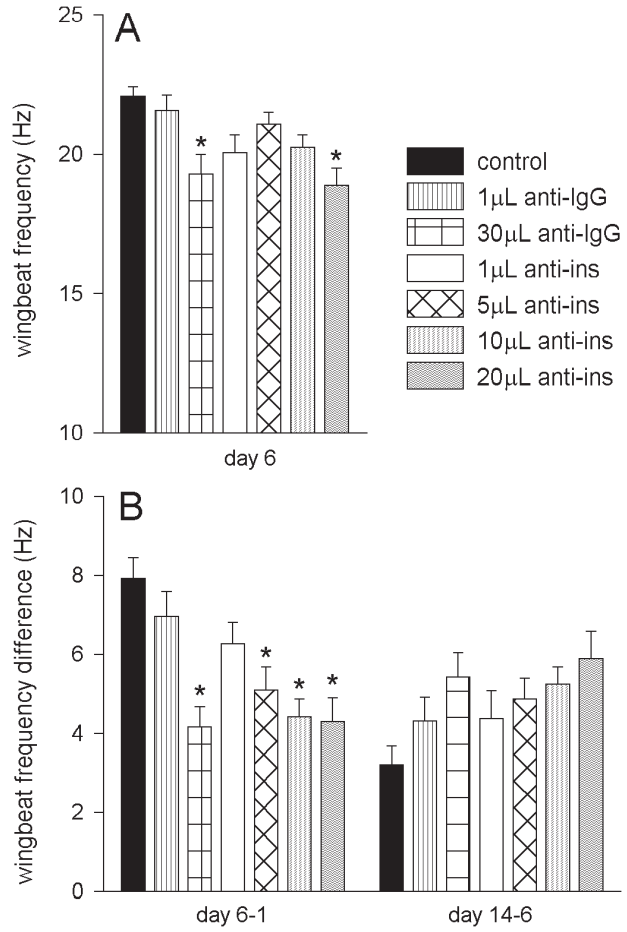
Fig. 1. Adult male locusts received daily injections of anti-insulin (anti-ins) or anti-IgG beginning the day of adult ecdysis (day 0). Beginning the day of ecdysis, 68 male locusts were injected daily with one of: 10 μL saline (n = 10); 1 or 30 μL anti-IgG (n = 10 and 9, respectively); 1, 5, 10, or 20 μL anti-insulin (n = 10, 10, 10, and 9, respectively). Where necessary, the antisera were diluted with PBS so the minimum volume injected was 5 μL. (A) The wingbeat frequency (WBF) of the locusts in the middle of adult maturation (day 6) were different from the control locusts only for the 30 μL anti-IgG and 20 μL anti-insulin treated locusts. (B) When the difference in WBF between day 6 and day 1 and day 14 and day 6 was calculated, the average increase in WBF was less than the control value for all doses of the antibodies (except for the 1 μL doses between day 1 and day 6). During the second half of maturation the control locusts showed the smallest increase in WBF, while the antibody treated locusts had larger increases in WBF.



the hindwing tegulae, a bilateral pair of proprioceptive organs, and then recovers toward the pre-ablation frequency during the next two weeks. This recovery is mediated by sprouting of the forewing tegulae afferents, which form new synaptic connections with flight system neurons and take over the role previously subserved by the hindwing tegulae (Büschges et al. 1992; Wolf and Büschges 1997).

We tested the hypothesis that insulin-related peptides are involved in mediating neuronal plasticity in the locust by attempting to manipulate the changes in WBF associated with maturation and functional recovery of the locust flight circuitry by manipulating levels of circulating insulin-related peptides using anti-insulin and (or) insulin. In light of the established role for insulin-related peptides in neuronal growth and development we expected to find significant differences in our WBF measures between anti-insulin and (or) insulin treated locusts and controls.

Materials and methods

Animals

Adult male locusts (*Locusta migratoria*) were obtained from a crowded colony maintained at Queen's University and were cared for in accordance with the guidelines of the CCAC. Tegulae were ablated either by cauterizing with a fine soldering tip or by pinching off the tegulae with a fine pair of forceps; these techniques have been shown to have the same effect on the locust flight system (Gee and Robertson 1996).

Measuring wingbeat frequency

Each locust was affixed by the pronotum to a rigid tether with a drop of hot wax. The locust was suspended in a wind tunnel (wind speed, ~2.5 m/s). The frequency of flashing of a stroboscope was adjusted until the wings appeared still and this frequency was taken to be the WBF. Locusts can couple their wingbeats to a stroboscope flashing at or near WBF, but in our experiments the stroboscope did not change WBF when measured electromyographically (Gee and Robertson 1996).

Measuring central flight rhythm frequency

We used a standard semi-intact preparation of the locust. Briefly, a dorsal incision was made and the thoracic ganglia were exposed. All thoracic nerve roots except nerve 1 were cut to ensure that there was no rhythm-dependent afferent feedback to the central flight circuitry from the flight system proprioceptors. A fine (100 μm) teflon-coated copper wire, insulated except at the tip, was placed into one of the dorsal longitudinal muscles to obtain an electromyographic (EMG) recording of the output frequency of the central flight circuitry. Flight rhythms were elicited by blowing through a hose aimed at the front of the locust's head and the frequency was obtained from the average of 4–5 successive cycles taken from the middle of 3–5 flight rhythm sequences for each locust.

Antibodies

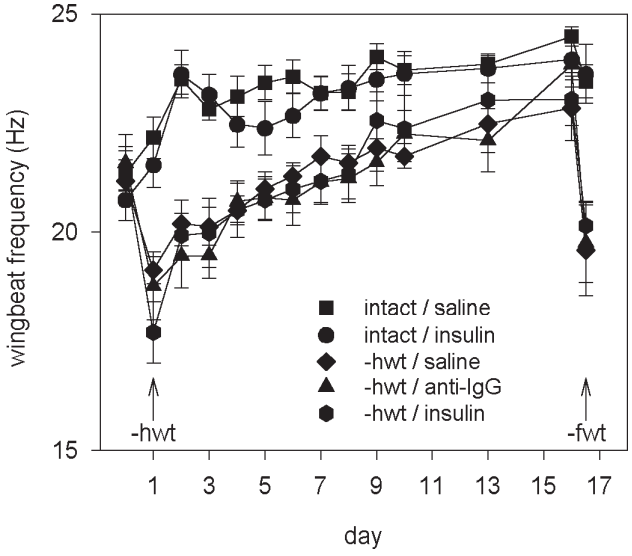
Antisera, insulin, and protein A-Sepharose 4B beads were purchased from Sigma Chemical Co. (St. Louis, Mo.). Whole antiserum against bovine insulin developed in guinea pig and delipidized whole serum developed in rabbit against bovine IgG were usually prepared by dialyzing against phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) to remove sodium azide prior to injection.

For one trial the antibodies were affinity purified using a Protein A-Sepharose 4B column following a low salt purification. Fractions containing IgGs were detected by reading the absorbance at 280 nm. IgG fractions were pooled and dialyzed against PBS then concentrated using a vacuum centrifuge. The protein concentration was determined using OD_{280} = 0.8 mg/mL. The final concentration was adjusted to 4.6 μg/μL using PBS.

Insulin

A stock solution of bovine insulin was prepared in distilled H_2O by adding a small amount of acetic acid until the insulin was solubilized (pH 4–5, 10 μg/μL). Before injecting, the stock solution was diluted with PBS to the appropriate concentration and to

Fig. 2. Mature adult locusts were injected twice daily with either saline, anti-IgG, or bovine insulin. Male locusts (n = 49) were divided into five groups which were treated as follows: 11 intact locusts were injected with 10 μL saline daily; 10 intact locusts were injected daily with 1 μg (in 10 μL) bovine insulin; 9 locusts had the hindwing tegulae ablated and received daily injections of 10 μL saline; 10 locusts had the hindwing tegulae ablated and received daily injections of 10 μL anti-IgG; and 9 locusts had the hindwing tegulae ablated and received injections of 1 μg (in 10 μL) insulin. The hindwing tegulae were ablated from three groups of locusts (–hwt) and wingbeat frequency subsequently decreased. Over the next two weeks, the WBF of the locusts with the ablated hindwing tegulae increased toward the WBF of the intact locusts. There was no difference in recovery between the control locusts and either the insulin or anti-IgG treated locusts. When the forewing tegulae were ablated from all locusts (–fwt) the WBF decreased in the locusts that had recovered from ablation of the hindwing tegulae and was unaffected in the intact locusts. There was also no difference in the WBF due to treatment after forewing tegulae ablation (–fwt).



pH 7.2. We injected 1 μg insulin in a 10 μL volume as the expected effective dose (equivalent to 0.55–0.9 mg insulin/g).

Statistical analyses

All values in the text and in the figures are mean ± standard error of the mean (SEM) unless otherwise indicated. Appropriate parametric and non-parametric tests were performed with the aid of SigmaStat statistical software (Jandel Scientific, San Rafael, Calif.). Significance was assumed at $P < 0.05$.

Results

Effect of injecting anti-insulin and anti-IgG on maturation of the locust flight system

In all trials we noted a difference in the coloration and hardness of the cuticle between the saline-injected and anti-insulin injected locusts. Anti-insulin injected locusts were paler and had softer cuticles than their saline-treated counterparts; the appearance of the anti-IgG-treated locusts fell in between these two groups.

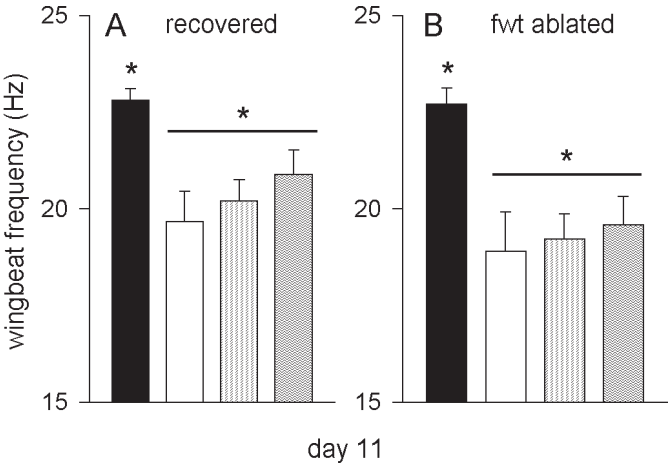
To investigate the effect of anti-insulin on the maturation of tethered intact flight, we injected different doses of anti-insulin in two separate trials. The results of one trial are shown in Fig. 1 and similar results were obtained from the other trial. WBF and weights were recorded on days 1, 6, and 14. On day 6 the mean WBF recorded from the locusts treated with the highest doses of both anti-insulin and anti-IgG injected were lower than the mean WBF of the saline-injected locusts (Fig. 1A). To get a measure of the effect of the various doses on maturation of WBF, we calculated the difference between successive measurements of WBF for each locust. There was a significant effect of the treatments on the maturation of WBF from day 1 to day 6. The mean WBF increase from day 1 to day 6 of all the anti-serum treated locusts, except the locusts receiving the 1 μL doses of both the anti-insulin and the anti-IgG, was significantly lower than the increase in WBF of the saline-injected locusts (Fig. 1B). Between day 6 and day 14 an almost mirror-image trend was seen and the mean increases in WBF were now greater for the anti-sera treated locusts than for the saline-injected locusts, although the differences between groups were not statistically significant. Several other attempts to manipulate WBF with anti-insulin by recording WBF daily and (or) administering anti-insulin every 12 h confirmed that there was no difference in WBF maturation between the anti-insulin and anti-IgG groups (data not shown).

We investigated whether there was an effect of the anti-insulin treatments on the output of the central flight circuitry and on growth of the thoracic nervous system (i.e., thoracic ganglion size). Eleven locusts were not treated, 11 were injected every 12 h with 10 μL anti-IgG and 10 were injected every 12 h with 10 μL of anti-insulin beginning 2 to 8 h after imaginal ecdysis. There were no differences in the output frequency of the de-afferented flight rhythm generator (in Hz ± SEM: control, 10.04 ± 0.21; anti-IgG, 9.95 ± 0.34; anti-insulin, 10.16 ± 0.26). However, ganglia from the anti-insulin treated locusts were smaller than the ganglia from both the anti-IgG and untreated locusts, which were not different from each other (e.g., for metathoracic ganglion length in mm ± SEM: control, 1.28 ± 0.04; anti-IgG, 1.24 ± 0.04; anti-insulin, 1.10 ± 0.02; measured using a compound microscope fitted with a calibrated ocular graticule).

Effect of insulin and anti-insulin on recovery of the flight system

We tested whether recovery of the locust flight system would be slowed by administration of anti-insulin or enhanced by insulin as recovery is mediated by neuronal growth (Büschges et al. 1992). The results from two series of hindwing tegulae ablations are presented. In the first instance, WBF was measured 14 days after imaginal ecdysis (day 0, Fig. 2). WBF was recorded during the next two weeks until day 16. After recording the WBFs on day 16, the forewing tegulae were ablated from all locusts and WBF was measured a second time several hours later. The WBF decreased after the hindwing tegulae ablations (Fig. 2). During the next two weeks, the flight system recovered with a gradual increase in WBF of the ablated locusts toward the WBF of the intact locusts. When the forewing tegulae were

Fig. 3. (A) There was no effect of injecting 55 μg of the purified IgG fraction of anti-insulin or anti-IgG on recovery of wingbeat frequency (WBF) on day 11 after hindwing tegulae ablation. (B) There was also no difference in the WBF due to treatment after forewing tegulae ablation.



ablated from all the locusts, there was a second decrease in the WBF of the locusts that had the hindwing tegulae ablations; there was little or no change in the WBF of the otherwise intact locusts. This pattern of recovery is the same as previously described (Gee and Robertson 1996). There was a significant effect of treatment, however, the differences were between the intact groups and the groups with hindwing tegulae ablations, and there was no effect of injecting either insulin or anti-IgG on WBF after the hindwing tegulae were ablated. There was also no difference between the intact locusts that were injected with saline and those injected with insulin (Student-Neuman-Keuls, $P > 0.05$).

The effects of administering injections of purified IgG fractions of the antisera on recovery from hindwing tegulae ablations were examined. This test was carried out to ensure that other proteins in the antisera were not interfering with the possible actions of the anti-insulin on recovery of the flight system. WBF was recorded from all locusts 11 days after the hindwing tegulae were ablated or sham-operations were performed. Daily injections of 55 μg (in 12 μL) of the IgG fractions of either anti-insulin or anti-IgG had no effect on the WBF of locusts recovering from hindwing tegulae ablations, although the WBF of the sham-operated locusts was significantly higher than all the ablated locusts (Fig. 3A). There was also no effect of the IgG injections on WBF after forewing tegulae ablation (Fig. 3B).

Discussion

Locusta insulin-related peptide(s) (LIP; Lagueux et al. 1990; Hetru et al. 1991) is a hypolipaemic hormone that is involved in oogenesis and embryogenesis. The C-peptide of LIP has direct effects on the membrane conductances of neurons isolated from locust thoracic ganglia suggesting physiological roles in the central nervous system (Bermudez et al. 1991), however long term effects of LIP on neural growth and differentiation are unknown. There is ample precedent for heterologous ligand binding of vertebrate insulin with invertebrate insulin-like receptors (e.g., Petruzelli et al. 1985; Jonas et al. 1996), though higher effective concentrations of the ligand are to be expected. Indeed, human insulin is capable of stimulating proliferation and neural differentiation in a *D. melanogaster* cell line (Pimentel et al. 1996), confirming a suggestion that the signal-transducing abilities of the insulin receptor has been conserved from invertebrates to mammals (Yamaguchi et al. 1995). The plasma half-life of injected insulin is short (less than 10 min in humans) and although a daily injection of insulin can be sufficient to trigger longer-lasting downstream events in the nervous system (e.g., Hayase and Yokogoshi 1995), our negative results from insulin treatment in locusts must be interpreted cautiously. We are more confident with the interpretation of the anti-insulin experiments.

We found a reproducible effect of injecting immature adult locusts with anti-insulin on qualitative observations of cuticular hardness and pigmentation. We could differentiate between the anti-insulin, the anti-IgG, and the untreated or saline-injected control locusts using these characteristics, especially if we also examined the flight muscle and the thoracic ganglia. We found both the weight of the locusts and the size of the thoracic ganglia were reduced in the anti-insulin treated locusts compared with anti-IgG treated locusts, indicating that the anti-insulin slowed overall growth of the locusts. Anti-insulin treatment slowed maturation of the locust flight system to the same extent as anti-IgG treatment indicating that there was a non-specific effect of injecting proteins into locusts. There was no effect of either anti-insulin or anti-IgG injections on recovery of the locust flight system after hindwing tegulae ablation. Bovine insulin is effective at stimulating neurite outgrowth in cultured locust neurons (Vanhems et al. 1990) and mimics the hypolipaemic effects of LIP (Loughton 1987). Also, antibodies raised against mammalian insulin cross-react with LIP in locust corpora cardiaca and in median neurosecretory cells of the brain (Orchard and Loughton 1980) and interfere with normal growth and development of juvenile instars of *Rhodnius prolixus* (Sevala and Loughton 1992) and *Tenebrio molitor* (Sevala et al. 1993). We are confident that our anti-insulin treatments would have affected LIP-mediated processes and we therefore propose that insulin-related peptides are not involved in the neuronal growth and sprouting associated with maturation and functional recovery of locust flight circuits.

Maturation and recovery of the locust flight system have become important models for studying mechanisms of plasticity in neural circuits. We undertook this study to test the role of insulin-like peptides in these two processes. While we did not prevent either maturation or recovery of the flight circuit we did find that treating immature adult locusts with anti-insulin had a negative effect on overall growth, nervous system growth, and pigmentation. It is conceivable that a role for insulin-related peptides in neural plasticity evolved subsequent to their general roles in metabolism and organismal growth and thus is evident only in organisms more advanced than locusts. Our results failed to support the hypothesis that locust insulin-related peptide is involved in regulating or promoting the neuronal growth associated with maturation and functional recovery in the locust flight system.

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