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The *Drosophila* gap gene *giant* regulates ecdysone production through specification of the PTTH-producing neurons

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ABSTRACT

In *Drosophila melanogaster*, hypomorphic mutations in the gap gene *giant* (*gt*) have long been known to affect ecdysone titers resulting in developmental delay and the production of large (giant) larvae, pupae and adults. However, the mechanism by which *gt* regulates ecdysone production has remained elusive. Here we show that hypomorphic *gt* mutations lead to ecdysone deficiency and developmental delay by affecting the specification of the PG neurons that produce prothoracicotropic hormone (PTTH). The gt^1 hypomorphic mutation leads to random loss of PTTH production in one or more of the 4 PG neurons in the larval brain. In cases where PTTH production is lost in all four PG neurons, delayed development and giant larvae are produced. Since immunostaining shows no evidence for Gt expression in the PG neurons once PTTH production is detectable, it is unlikely that Gt directly regulates PTTH expression. Instead, we find that innervation of the prothoracic gland by the PG neurons is absent in *gt* hypomorphic larvae that do not express PTTH. In addition, PG neuron axon fasciculation is abnormal in many *gt* hypomorphic larvae. Since several other anteriorly expressed gap genes such as *tailless* and *orthodenticle* have previously been found to affect the fate of the cerebral labrum, a region of the brain that gives rise to the neuroendocrine cells that innervate the ring gland, we conclude that *gt* likely controls ecdysone production indirectly by contributing the peptidergic phenotype of the PTTH-producing neurons in the embryo.

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Introduction

The *giant* (*gt*) locus codes for a zinc finger containing transcription factor that is widely known for its role in specifying early anterior/posterior pattern in the blastoderm embryo (Capovilla et al., 1992; Eldon and Pirrotta, 1991; Reinitz and Levine, 1990; Stanojevic et al., 1991). Amorphic *gt* alleles lead to embryonic lethality due to the loss of posterior abdominal segments 5–7 and sometimes 8, as well as labral and labial structures in the head region. (Gergen and Wieschaus, 1985; Mohler et al., 1989; Petschek et al., 1987). However, the original *gt* alleles were discovered and partially characterized as mutations that produced large larvae as a result of developmental delay (Bridges and Gabritschevsky, 1928). These mutants played an important role in the early history of *Drosophila* genetics because they also produced larger than normal polytene chromosomes that aided early cytogenetic studies (Bridges, 1935).

The viable *gt* alleles exhibit variable penetrance for the developmental delay phenotype (~25% females, 13% males) that can be enhanced in females when placed over a deficiency, suggesting that they are hypomorphic mutations (Schwartz et al., 1984). These phenotypically giant larvae exhibit pronounced developmental delay especially during the third instar stage and pupate approximately 5 days later than wildtype (Schwartz et al., 1984).

Post-embryonic development in holometabolous insects is characterized by defined molting periods followed by metamorphosis. The precise timing of these events is regulated at a systemic level in response to multiple cues such as nutritional status, body size, organ development and environmental conditions (Edgar, 2006; Menut et al., 2007; Mirth and Riddiford, 2007; Nijhout, 2003). These cues likely regulate developmental timing in several ways, but ultimately they impinge upon the production and secretion of the insect steroid hormone ecdysone. In *gt* hypomorphic larvae, the protracted third instar stage appears to result from a delay in the rise of ecdysone titer that precedes the initiation of metamorphosis, since feeding these animals 20-hydroxecdysone (20-E) reverts the delay phenotype leading to normal size larvae that pupate at the appropriate time (Schwartz et al., 1984).

The molecular mechanism by which *gt* controls ecdysone production has been a long-standing mystery. In many insects, the regulation of ecdysone production in larvae involves two major components: a pair of bilaterally symmetric neurons (PG neurons) located in the cerebral labrum portion of the brain, and the prothoracic gland, the endocrine organ that actually produces and secretes ecdysone (Gilbert et al., 2002). In *Drosophila,* the PG neurons directly innervate the prothoracic gland (Siegmund and Korge, 2001) and induce production and secretion of ecdysone by releasing an adenotropic peptide hormone called

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prothoracicotropic hormone (PTTH) (McBrayer et al., 2007). PTTH signals through the receptor tyrosine kinase Torso to activate a RAS/ERK cascade that ultimately stimulates transcription of ecdysone biosynthetic enzymes (Rewitz et al., 2009). Intriguingly, elimination of PTTH signaling delays the rise in ecdysone titer and the onset of pupation by approximately 5 days resulting in large pupae and adults, similar to those produced by *gt* hypomorphs (McBrayer et al., 2007; Rewitz et al., 2009). The similarity in phenotype between *gt* hypomorphs and loss of PTTH signaling prompted us to investigate whether *gt* in some way controls PTTH signaling. Here we report that rather than directly regulating PTTH production in the PG neurons, *gt* indirectly controls PTTH and subsequent ecdysone production by influencing the development of the PTTH-producing PG neurons.

Materials and methods

Drosophila stocks and husbandry

The gt^1 and gt^{E6} lines were obtained from the Bloomington *Drosophila* stock center. gt^1*; ptth-HA* stocks were generated by standard genetic methods. Genomic *ptth-HA-50A* line (*yw; ptth-HA*) and the *yw; Feb211-Gal4; UAS-GFP* (Feb211-GFP) line were described previously (McBrayer et al., 2007; Siegmund and Korge, 2001). gt^1*;UAS-GFP; Feb211-Gal4* larvae were obtained by crossing Feb211-GFP males to gt^1 females and selecting male larvae. Flies were raised at 25 °C on standard food in vials.

Immunohistochemistry

The following antibodies were used at the indicated dilutions for immunohistochemistry: rat anti-HA 3F10 (Roche) 1/500, rat anti-Gt (generous gift from Dr. Vincenzo Pirrotta) 1/500 and mouse anti-CSP (Iowa Hybridoma Bank) 1/100. The Alexa series (Invitrogen) of secondary antibodies were used for immunofluorescence at 1/500 dilution. CNSs from third instar wandering larvae were dissected out and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature for anti-HA and anti-CSP staining. Antibody staining and washes of larval CNSs were conducted in 0.1% Triton-X100 in 1× PBS (PBST). Primary antibody treatments of CNSs were done at 4 °C for 24 h. Embryos were dechorionated in 50% bleach, fixed in 4% paraformaldehyde in PBS for 15 min and all subsequent antibody reactions were in PBS + 0.1% Tween-20. Samples were mounted in

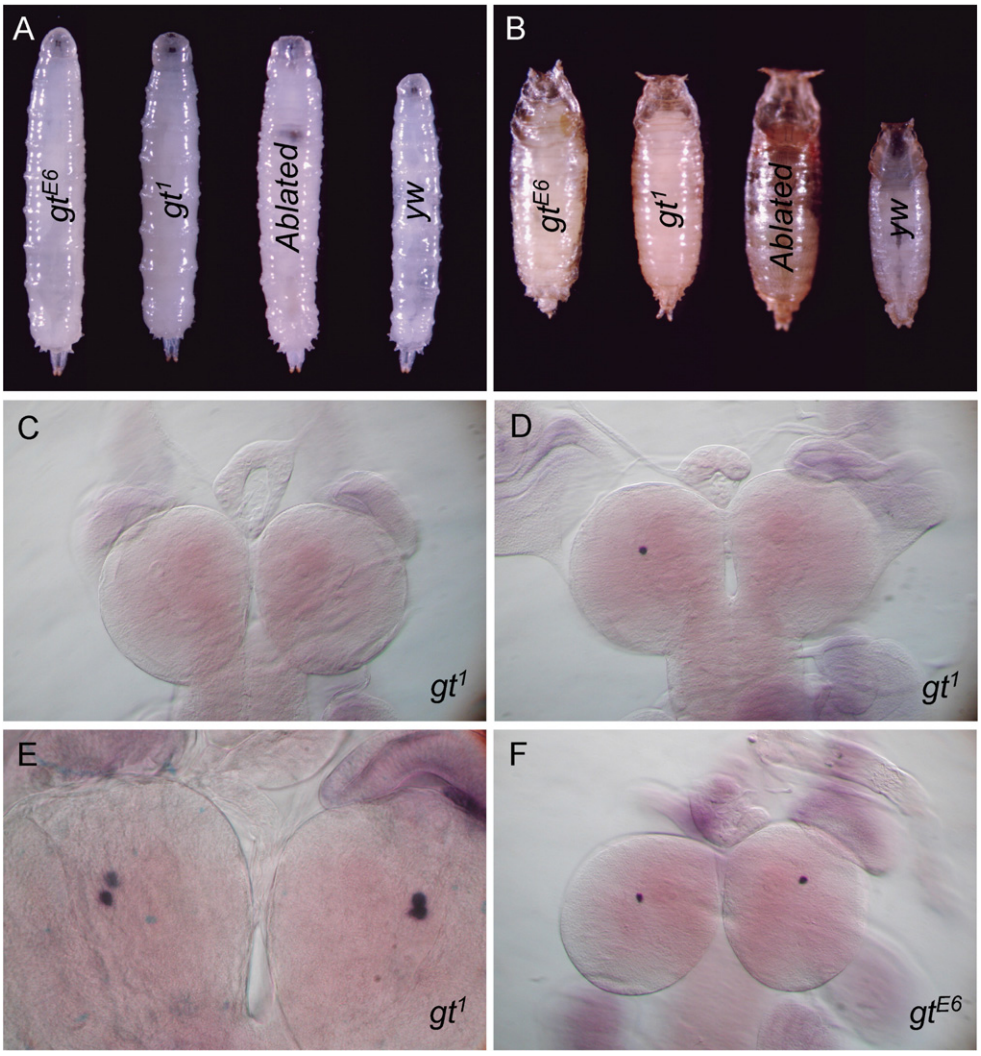

Fig. 1. Hypomorphic *giant* mutants are phenotypically similar to PG neuron ablated flies and show variable loss of PTTH expression in the PG neurons. gt^1 and gt^{E6} larvae exhibit a prolonged third instar stage in about 25% of females and 13% of males. This phenotype, although much less penetrant, is similar to PG neuron ablated larvae (*ptth*>Gal4/UAS-*grim*). This prolonged third instar stage gives rise to larvae and pupae that are significantly larger than *yw* control animals (A and B, pictures were taken at same magnification and settings and fused using Photoshop). Consistently, *ptth* expression, as observed by *in situ* hybridization, is lost from all four PG neurons in the developmentally delayed gt^1 larvae (C). Normally developing *gt* hypomorphic larvae show a variable stochastic loss of *ptth* expression ranging from one-three of the four PG neurons (D–E). Many of them express *ptth* in all four PG neurons similar to wild type animals (E) and most of these animals show no developmental delay.

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