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In developing *Drosophila* neurones the production of γ-amino butyric acid is tightly regulated downstream of glutamate decarboxylase translation and can be influenced by calcium

Barbara Küppers,[1] Natalia Sánchez-Soriano,[1] Johannes Letzkus, Gerhard M. Technau and Andreas Prokop

Institute of Genetics, University of Mainz, Mainz, Germany

Abstract

The presented work pioneers the embryonic *Drosophila* CNS for studies of the developmental regulation and function of γ-amino butyric acid (GABA). We describe for the first time the developmental pattern of GABA in *Drosophila* and address underlying regulatory mechanisms. Surprisingly, and in contrast to vertebrates, detectable levels of GABA occur late during *Drosophila* neurogenesis, after essential neuronal proliferation and growth have taken place and synaptogenesis has been initiated. This timeline is almost unchanged when the GABA synthetase glutamate decarboxylase (GAD) is strongly misexpressed throughout the nervous system suggesting a tight post-translational regulation of GABA expression. We confirmed such GABA control mechanisms in an independent model system, i.e. primary *Drosophila* cell cultures raised in elevated [K^+]. The data suggest that, in both systems, GABA suppression occurs via control of GAD activity. Using developing embryos and cell cultures as parallel assay systems for pharmacological and genetic studies we show that the negative regulation of GAD can be overridden by drugs known to elevate intracellular free [Ca^{2+}]. Our results provide the basis for investigations of genetic mechanisms underlying the observed phenomenon, and we discuss the potential implications of this work for *Drosophila* neurogenesis but also for a general understanding of GAD regulation.

Keywords: calcium, cell culture, embryonic development, GABA, GAD, synapse.

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Developing neurones grow towards their target cells and form synapses of the appropriate type. At these synapses, neurotransmitters mediate information transfer. In addition, there are indications that neurotransmitters also influence the development of neurones and neuronal networks. For example, in vertebrates the neurotransmitter GABA (γ-amino butyric acid) plays a role during proliferation and migration of neurones (Varju *et al*. 2001). Furthermore, manipulations of GABA levels or GABA receptors function modulate neurite growth (Fukura *et al*. 1996; Maric *et al*. 2001), the number of inhibitory synapses on Purkinje cells (Seil *et al*. 1994), and dendritic outgrowth and regression (Mattson and Kater 1989). Also the timeline of GABAergic transmission seems of importance. For example, in vertebrates GABAergic synapses develop prior to glutamatergic ones and display excitatory properties at these early stages (Ben-Ari *et al*. 1997). In chick embryos GABAergic transmission influences activity patterns of motorneurones whilst they grow towards their target muscles (Milner and Landmesser 1999). Hence, it seems that during vertebrate neurogenesis the quantitative, qualitative and temporal regulation of GABA pathway components is of importance.

We investigate mechanisms underlying the formation of neuronal circuits and synapses making use of the detailed cellular knowledge of the embryonic nervous system of *Drosophila melanogaster* and its genetic amenability

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Address correspondence and reprint requests to A. Prokop, Institute of Genetics, University of Mainz, J.-J.-Becherweg 32, D-55128 Mainz, Germany. E-mail: prokop@mail.uni.mainz.de

[1]Authors contributed equally.

Abbreviations used: ATP, adenosine triphosphate; GABA, γ-amino butyric acid; GAD, glutamate decarboxylase; SM5K, Schneider's *Drosophila* medium 5 mM potassium; SM40K, Schneider's *Drosophila* medium 40 mM potassium; SM80K, Schneider's *Drosophila* medium 80 mM potassium; PBT, phosphate-buffered saline with 0.1% Triton-X; PSC, post-synaptic currents; TTX, tetrodotoxin.

(reviewed in Prokop 1999). So far, little is known concerning neurotransmitter regulation and their potential influences on neurogenesis in *Drosophila* (and other invertebrates), and here we have begun to study the potential regulation and role of GABA. Components of the *Drosophila* GABA pathway studied so far are the GABA-producing enzyme glutamate decarboxylase 1 (GAD1; EC 4.1.1.15; Jackson *et al*. 1990), the chloride-conducting ionotropic GABA receptor resistance to dieldrin (RDL; Chen *et al*. 1994), and two $GABA_B$ receptor subunits (Mezler *et al*. 2001).

In this study, we investigate the role of GAD1 in the context of GABA regulation in *Drosophila*. Detectable levels of GABA occur late, not before onset of the formation of at least excitatory synapses. We show that this late appearance is ensured by negative regulatory mechanisms, which appear to act via suppression of GAD1 activity. To be able to study the meaning of this regulation for *Drosophila* neurogenesis we started to address underlying mechanisms. For this purpose we have established primary cell cultures as a second assay system for the study of GABA regulation. We show that commercial drugs known to elevate intracellular free $[Ca^{2+}]$ are sufficient to override GABA suppression in cell cultures and in developing embryos. As discussed, our data and the assay systems provided here have potential to contribute to a general understanding of GABA regulation in the context of development and neurological disease.

Materials and methods

Fly stocks

We used *elav-Gal4*^{C155} (courtesy of Bloomington stock centre; Luo *et al*. 1994), *Mz1407-Gal4* (Luo *et al*. 1994); *Uas-mCD8-GFP* (courtesy of L. Luo and Bloomington stock centre; Lee and Luo 1999) and *GAD1* deficiency *Df(3L)C175* (courtesy of F.R. Jackson; Kulkarni *et al*. 1994). The transgenic fly strains *Uas-GAD1*^{142} (2nd chromosome) and *Uas-GAD1*^{221} (3rd chromosome) were established following standard procedures (Spradling and Rubin 1982) with a pUAST-vector carrying a *Uas-GAD1* insert (vector kindly provided by F.R. Jackson; Featherstone *et al*. 2000). Flies carrying both *Uas-GAD1* insertions were used for our experiments. Further fly strains are listed in Table 1.

Primary cell cultures

As described previously (Schmidt *et al*. 2000), cells were removed with glass capillaries from 50 to 100 embryos at gastrula stage and transferred to culture medium to wash debris away. After gentle spin supernatant was removed, cells were resuspended in culture medium (5 μL/donor embryo) and aliquots of 30–40 μL were transferred to flat-bottom wells of glass slides. Wells were sealed air-tight with a greased coverslip, kept at 26°C, and turned upside down after 2–24 h (hanging drop cultures). Schneider's *Drosophila* media (Schneider 1964) were obtained from Gibco (Rockville, MD, USA) or produced by ourselves. $[K^+]$ and $[Ca^{2+}]$ were adapted via KCl or $CaCl_2$ content, respectively. After fetal calf serum (20%, non-heat-inactivated; Biochrom, AG Seromed, Berlin, Germany) was added, medium was kept for 3 days in the dark at 26°C, then insulin

Table 1 Genetic tests of GABA expression

Genotype	Reference	GABA at 21 h	Comment
a Suppressing transmission			
Dunc-13^{P8420}	1	+	Abolishes evoked transmission and reduces frequencies of miniature events
syntaxin^{229}	2	+	Abolishes all transmission
n-synaptobrevin$^{\Delta F33B}$	3	+	Abolishes evoked transmission and reduces frequencies of miniature events
elav::tetanus toxin G	4	+	Cleaves n-Synaptobrevin in all neurones
elav::tetanus toxin G::GAD1	–	+	Ectopic GABA from co-expressed GAD1 is not suppressed by tetanus toxin
choline acetyl transferase^{ts1}	5	+	Fails to produce acetyl choline when raised at 29°C throughout development
b Suppressing action potentials (AP)			
paralytic$^{Df(1)LD34}$	6	+	Abolishes AP when kept at 34°C throughout development and dissection
elav::Kir2.1-GFP	7	+	Abolished AP via pan-neuronal expression of inward rectifying K^+ channel
elav::Kir2.1-GFP::GAD1	–	+	Ectopic GABA from co-expressed GAD1 is not suppressed by Kir2.1-GFP
c Manipulating calcium			
Caα1D^{X7}	8	+	Voltage-dependent Ca^{2+} channel; loss of function
paralytic$^{Df(1)LD34}$*; Caα1D*^{X7}	–	+	Double mutant for paralytic and *Caα1*^{DX7} Ca^{2+} channel
nightblind^{HF368}	9	+	Voltage-dependent Ca^{2+} channel subunit; loss of function
nightblind^{HF368}*; Caα1D*^{X7}	–	+	Combined loss of function of two Ca^{2+} channel subunits

'Genotype': lists genetic constellations and alleles used; 'Reference': stock details at the bottom of the legend; 'GABA at 21 h': indicates normal (+) GABA staining in the stage 17 nerve cord; 'Comment': explains the nature of the genotype and lists further information. (1) by courtesy of K. Broadie, Aravamudan *et al.* (1999); (2) by courtesy of H. Bellen, Broadie *et al.* (1995); (3) by courtesy of C. O'Kane, Yoshihara *et al.* (1999); (4) by courtesy of S. Sweeney, Sweeney *et al.* (1995); (5) by courtesy of R. Baines, Baines *et al.* (2001); (6) by courtesy of Bloomington stock centre, Broadie and Bate (1993b); Baines and Bate (1998); (7) by courtesy of R. Baines, Baines and Bate (1998); (8) by courtesy of Bloomington stock centre, Eberl *et al.* (1998); (9) by courtesy of Bloomington stock centre, Smith *et al.* (1996).

(2 μg/mL; Sigma, St Louis, MO, USA) was added and pH set to 6.8–6.9. Maximal use of culture media was 21 days.

Immunohistochemistry

Embryos were dissected flat in external bath solution following standard protocols (Broadie 2000). Dissected embryos and cultured cells were fixed 30–60 min in 4% paraformaldehyde in 0.05 M phosphate buffer (pH 7–7.2), washed for 1 h in phosphate-buffered saline with 0.1% Triton-X (PBT). Incubation with antibodies was carried out in PBT without blocking reagents. We used antibodies raised against GABA (rabbit, 1 : 1000, Sigma), rat GAD (1440 from sheep, 1 : 500; courtesy of J. Harvey-White, from a source developed at NIH, Bethesda, MD, USA; Oertel *et al*. 1981), *Drosophila* GAD1 (rabbit, 1 : 500; courtesy of F.R. Jackson; Featherstone *et al*. 2000), Synapsin (mouse, 1 : 10; courtesy of E. Buchner; Klagges *et al*. 1996), CD8 (rat, 1 : 10; Caltag Laboratories, Burlingame, CA, USA), Elav (Elav-7E8A10 from rat, 1 : 1000; Developmental Studies Hybridoma Bank, The University of Iowa, IA, USA; O'Neill *et al*. 1994), and fluorochrome-coupled secondary antibodies (Jackson Immuno-Research, West Grove, PA, USA; dilution 1 : 200).

Electrophysiology

As described previously (Schmidt *et al*. 2000), whole-cell patch-clamp recordings of primary cell cultures were performed at room temperature after 20–90 h *in vitro*, between 1 and 6 min after establishing whole-cell configuration (no significant rundown observed). The intracellular solution contained: 133 mM CsOH, 133 mM methane sulphonate, 5 mM CsCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES, 4 mM adenosine triphosphate (ATP) and 0.5 mM guanosyl triphosphate, pH 7.2. The external solution contained: 100 mM NaCl, 4.6 mM KCl (for SM5K cultured cells) or 40 mM KCl (for SM40K cultured cells), 2 mM $CaCl_2$, 2 mM $MgCl_2$, 84.7 mM or 14 mM sucrose and 10 mM HEPES, pH 7.2. The liquid junction potential of 10 mV generated in these solutions was corrected online. Data were recorded at −75 mV. Signals were amplified, filtered at 2 kHz (4-pole Bessel) and sampled at 20 kHz on a Macintosh computer using an EPC-9 amplifier and Pulse (HEKA, Lambrecht/Pfalz, Germany). Cell capacitances were 5.9 ± 2.3 pF, input resistances 1.32 ± 1.09 GΩ. Cells with input resistance < 0.8 GΩ were discarded. The voltage error due to series resistance was always < 10 pA. Event frequencies and amplitudes were analysed using Mini Analysis (Synaptosoft, Decatur, GA, USA) with threshold criteria of 10 pA for amplitude (rms noise < 2.5 pA) and 4 fC for charge transfer. Single channel activity was rejected during data inspection. Drugs (1 μM tetrodotoxin, 1 μM α-bungarotoxin) were bath applied.

Results

GABA occurs late during embryonic development in a subpopulation of neurones

To determine when GABA function is required during development, we analysed the temporal and spatial pattern of GABA immunoreactivity in wild-type embryos and freshly hatched larvae. At the time of hatching, when the number of embryonic nerve cells is complete and neurones have acquired mature electrical properties (Baines and Bate 1998), GABA is exclusively expressed in the CNS. In the ventral nerve cord it is found throughout the neuropile (synaptic region), and particularly strong staining is seen in segmentally repeated patterns in the cortex (cell body area) but not dorsal of the neuropile and at the dorsal midline (asterisks in Figs 1a and d′). Strongly expressing cells predominate in the periphery of the cortex, whereas cells in more central positions show relatively lower levels of GABA staining. Cell counts revealed on average 27 strongly and 25 weakly labelled GABA-positive neurones on either side of each segment. Thus, about 20% of all neurones show GABA immunoreactivity (in total approximately 250 neurones/hemisegment; Schmidt *et al*. 1997). Two different antibodies against the GABA-producing enzyme GAD1 stain a very similar population of neurones as anti-GABA antibodies (Figs 1g and 3c). The staining of both anti-GABA and anti-GAD antibodies is eliminated in embryos lacking the *GAD1* gene (Figs 1f and h), demonstrating their specificity and suggesting that all detectable GABA is due to GAD1 activity (see also Featherstone *et al*. 2000). Double-labelling with anti-Elav antibodies suggests that GABA is restricted to neurones (Figs 1a′–c′). Double labellings with anti-Engrailed antibodies suggest that some GABA-positive neurones at the ventral midline are VUM-interneurones (data not shown; see Bossing and Technau 1994).

Having described the GABA expression pattern in the mature embryo we next analysed the timeline of GABA expression. CNS development in *Drosophila* embryos can be roughly subdivided into three partially overlapping phases: a phase of birth and specification of neurones (ending at about 14 h), a phase of major axonal outgrowth (9–13 h), and a stage of synapse formation and neurite refinement (13–21 h). We could not detect GABA before 16 h of development (Figs 2c′ and 3d–f). At this time, synaptogenesis in the CNS has started as suggested by the localization of the pre-synaptic marker Synapsin (Fig. 2c) and reports of first synaptic events and co-ordinated movements (Fig. 2a; Baines and Bate 1998). Initial GABA immunoreactivity is weak and relatively homogeneous, whereas after 17 h an increasing number of cell bodies shows higher levels of GABA immunoreactivity (Figs 2d′ and 3g). Thus, GABA staining appears in a gradual fashion, some hours after first excitatory synaptic transmission occurs (Baines and Bate 1998).

Onset of detectable GABA production in the embryonic CNS is controlled downstream of GAD1 translation

The late onset of GABA production is surprising because increasing GAD1 immunostaining is detectable from 12 to 13 h, i.e. about 4 h before the onset of faint GABA staining (Figs 3a and b; Featherstone *et al*. 2000). This late appearance of GABA might be due to the initially low

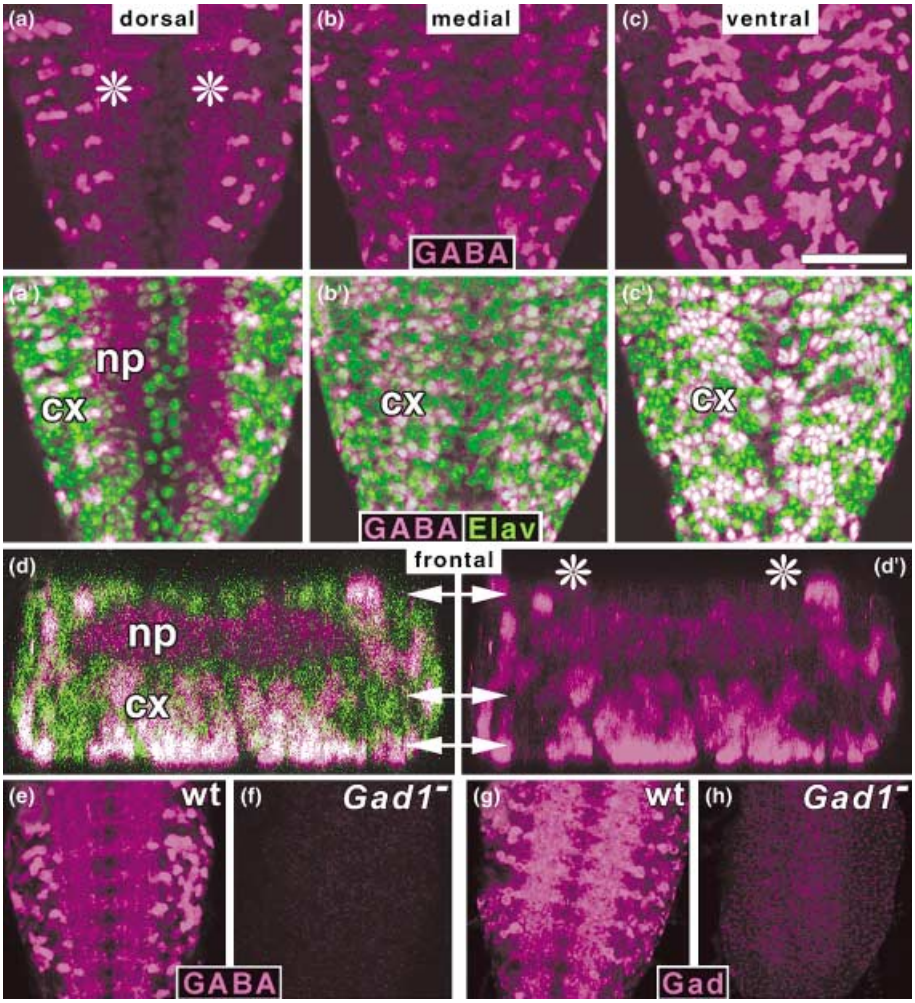

Fig. 1 Expression pattern of GABA in late embryos. Ventral nerve cords of late stage 17 embryos (anterior up; cx, cortex; np, neuropile); primed and unprimed versions of the same letters indicate identical views of the same specimens. (a–c′) Dorsal, medial and ventral horizontal confocal sections of one specimen labelled with antibodies against GABA (a and c) and GABA plus Elav, a protein specific to neuronal nuclei (a′, b′ and c′ asterisks indicate area without GABA-positive cells). (d and d′) Frontal view (confocal Z-stack) of the same specimen [double arrows indicate horizontal positions of (a) to (c′)]. (e–h) Anti-GABA or anti-GAD staining in late embryonic wild-type and *GAD1* mutant nerve cords (anterior top) indicating specificity of used antisera. GABA staining in the neuropile appears weak as sensitivity levels of the confocal microscope had to be reduced due to extremely high staining intensity in cell bodies. Scale bar 20 μm (a–c′), 13 μm (d and d′), and 30 μm (e–h).

amount of its synthetase GAD1. To test this we tried to express GAD1 at higher levels, using transgenic flies carrying a Gal4-inducible *GAD1* gene (see Materials and methods). The use of *Uas-GAD1* flies allows targeted expression of *GAD1* when crossed to transgenic flies expressing the yeast transcription factor Gal4, which specifically binds to the Uas sequence and activates the linked genes (Brand and Perrimon 1993). Accordingly, high levels of GAD1 can be detected throughout the whole nervous system already at 12 h of development (Fig. 3a′) when *Uas-GAD1* is combined with the pan-neuronal Gal4 strains *Mz1407-Gal4* or *elav-Gal4*. As a result, strong ectopic GABA staining is seen in late embryonic nervous systems (Fig. 3h′), including the peripheral neurones (Fig. 3, white arrows) and neuromuscular junctions (Fig. 3j, arrow heads). Interestingly, in wild-type embryos, GABA is not detectable at neuromuscular junctions in spite of the fact that GAD1 activity exists in motorneurones and has been shown to reduce glutamate levels (Featherstone *et al.* 2000, 2002). Therefore it is possible that GABA accumulation is prevented at the wild-type neuromuscular junction by either enzymatic degradation or by release. Our present results demonstrate that such mechanisms preventing GABA accumulation seem to be little efficient and can be overcome by overexpression of GAD1.

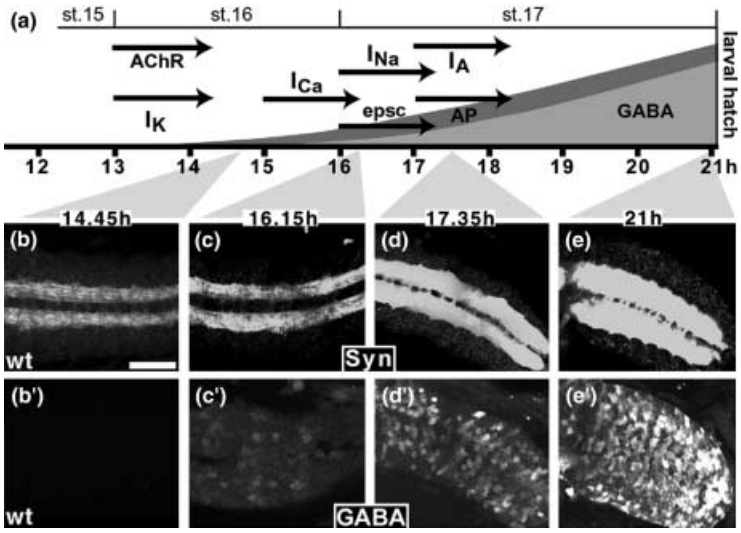

Fig. 2 Timeline of GABA expression. (a) Developmental timeline (bottom, hours of embryonic development; top embryonic stages according to Campos-Ortega and Hartenstein 1997) illustrating the increase of GABA staining in wild-type (medium grey; see b′-e′ and Figs 3d–h) and embryos with pan-neuronal GAD1 expression (dark grey; compare Figs 3d′–h′), and periods of first occurrance of certain electrical properties (arrows; taken from Baines and Bate 1998): AChR, acetyl choline receptiveness; AP, action potentials; epsc, excitatory postsynaptic currents; I_A, fast-inactivating K^+ current; I_{Ca}, Ca^{2+} currents; I_K, slow-inactivating K^+ currents; I_{Na}, Na^+ currents. (b–e′) Ventral nerve cords (anterior left) at different age (hours after egg lay is indicated) double stained for the pre-synaptic marker Synapsin (b–e) and GABA (b′–e′); essential GABA staining occurs after strong Synapsin staining (i.e. synapse formation) has been established. Scale bar 20 μm.

Thus, in the late embryo, pan-neuronal expression of GAD1 is sufficient to produce GABA in most if not all neuronal cells. However, neurones of 13-hour-old embryos (Fig. 3d′) show no or very faint GABA staining, even when embryos were raised at 29°C (increased temperature enhances Gal4 activity). At 15–16 h, GABA levels increase homogeneously (Figs 3e′ and f′). However, brightly stained cells occur only in older embryos, e.g. at 17 h (Fig. 3g′). This temporal pattern of GABA, upon pan-neuronal expression of *GAD1*, is remarkably similar to that observed in wild-type embryos (compare Figs 3d–h and 3d′–h′).

In summary, our experiments show that GAD1 is absolutely required for the production of detectable GABA levels (Fig. 1f), but its presence is not sufficient before a key developmental stage, indicative of regulation downstream of *GAD1* translation. This regulation seems independent of synaptic release as neurones at 13 h do not yet show synaptic activity (Baines and Bate 1998). Furthermore, it appears mostly independent of GABA catabolism: This is suggested by observations at the neuromuscular junction (see explanation above) and by the fact that many non-GABAergic neurones with targeted GAD1 expression fail to upregulate GABA, although they would not be expected to harbour highly efficient GABA-catabolizing enzymes. Because also the required substrate glutamate is present at earlier stages (Broadie and Bate 1993a), we conclude that GAD1 activity itself is inhibited in earlier embryos, potentially sharing common features with post-translational control of vertebrate GAD (see Discussion). Interestingly, the suppression of GAD1 activity covers a period when essential neuronal growth and synaptic differentiation take place and it is therefore tempting to speculate that this tight regulation might be of developmental significance.

GABA expression in primary cell cultures is influenced by $[K^+]$

We observed regulation of GAD1 activity also in primary cell culture. This cell culture develops from neural precursor cells obtained from *Drosophila* embryos at the gastrula stage. Raised at 26°C in serum-supplemented Schneider's *Drosophila* medium (Schneider 1964) these cultured precursor cells give rise to cell lineages with cell numbers comparable to those of precursor cells in the nervous system (K. Lüer and G. Technau, unpublished data). These cells develop projections and electrical properties typical of *Drosophila* neurones (Schmidt *et al*. 2000; see below). However, the staining intensity and frequency of GABA-positive cells is low (approximately 3%; visible after about 40 h *in vitro*). This poor GABA expression was improved by lowering $[K^+]$ from 21.8 mM in serum-supplemented Schneider's medium to 4.6 mM (referred to as SM5K). In SM5K, the time course of GABA expression is unchanged, but the frequency of cells containing GABA increases to 15.6 ± 3.2%, many of them at high levels (Fig. 4a). This percentage is comparable to the estimated 20% of GABA-positive cells in the ventral nerve cord (see above). In contrast, if $[K^+]$ in the medium is

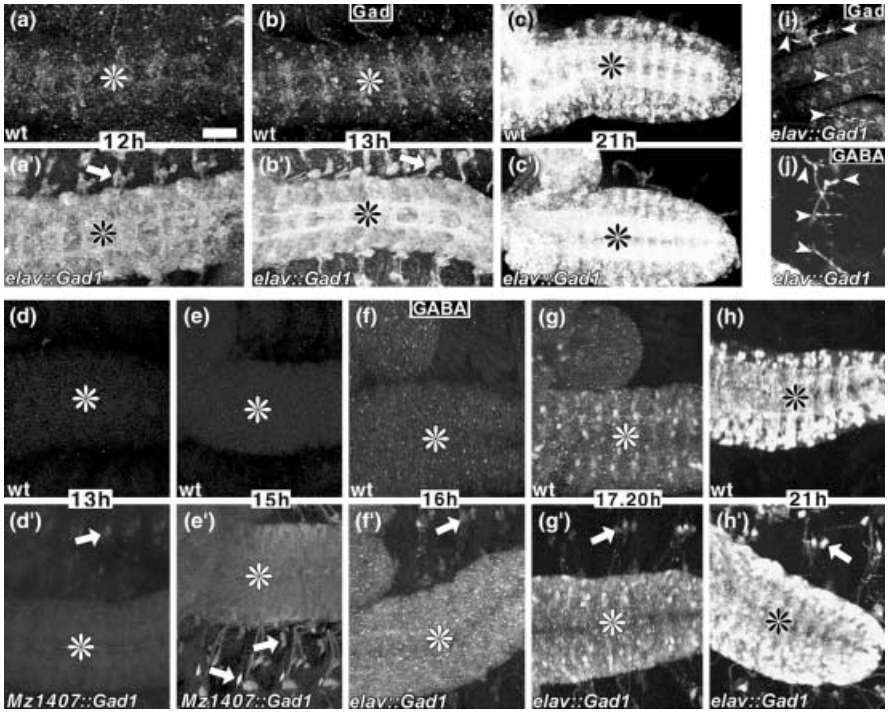

Fig. 3 Misexpression of GAD1 has only little impact on the GABA timeline. (a–c) In the ventral nerve cord (asterisks; anterior left) of wild-type embryos GAD1 immunoreactivity becomes first visible at 12–13 h. (a′–c′) Upon pan-neuronal expression of *GAD1* (*elav::GAD1*) GAD1 immunoreactivity is significantly increased and occurs much earlier. (d–h′) Comparing ventral nerve cords of wild-type (d–h) with those of embryos with pan-neuronal expression of GAD1 (*elav::GAD1* or *Mz1407::GAD1*; both give comparable results; d′–h′) reveals little difference in the GABA timeline, and strongly stained cells in the CNS can first be seen in the 17- to 21-hour-old embryos in both cases (g and g′). Whereas in 21-hour-old wild-type embryos GAD1 or GABA immunoreactivity are restricted to the CNS [asterisks in (a–c) and (d–h)], immunoreactivity with both antisera can be found in sensory neurones [white arrows in (a′ and b′) and (d′–h′); in (c′) not in focus] and motor terminals [arrowheads in (i) and (j); staining in muscles in (i) is background] when GAD1 is misexpressed pan-neuronally. Scale bar 10 μm in (i) and (j) and 20 μm in all others.

increased to 40 mM or 80 mM (referred to as SM40K or SM80K, respectively) the number of GABA-expressing cells is reduced to less than 1% and the staining intensity is weak (Fig. 4b). Because the morphology of neurones cultured under these conditions appears normal (see Fig. 6a′), GABA reduction appears not to be a secondary effect of cell death or stress. Indeed, physiological concentrations of up to 80 mM [K^+] have been reported for *Drosophila* (Stewart *et al*. 1994 and references therein).

Suppression of GABA production in neurones cultured in high [K^+]-containing media occurs downstream of GAD1 translation

We tested whether the regulation of GABA production occurs downstream of *GAD1* translation. Unfortunately, the levels of GAD1 protein cannot be monitored directly as the two anti-GAD antisera used for embryos (Figs 1g and 3a–c′) cause severe background staining in our cell cultures using various fixation and staining procedures (paraformaldehyde, methanol, picric acid, heat, absence of detergent, different secondary antibodies; data not shown). To ensure that the cultured neurones contain GAD1, we expressed *GAD1* in cell cultures derived exclusively from donor embryos expressing GAD1 throughout the nervous system (*Uas-GAD1* driven by the pan-neuronal Gal4 line *elav-Gal4*). When these cultures are raised in SM5K, GABA is produced in 53.5 ± 18.9% of cells, thus about half of all cells without intrinsic GABA production respond positively to targeted *GAD1* expression (Fig. 4a; most further experiments were carried out with *elav::GAD1* cells). However, when growing the cells in SM40K or SM80K for 3–4 days, GABA frequencies were below 1%, similar to that in cell culture without ectopic *GAD1* (Figs 4b and 6a′). This suppression of GABA staining in SM40K or SM80K is unlikely to be due to inhibition of *GAD1* expression since the Gal4/Uas-system was designed to circumvent intrinsic gene regulation (Brand and Perrimon 1993). To confirm this, *Uas-GAD1* was co-expressed with a *Uas-mCD8-GFP* reporter gene (Lee and Luo 1999) in the same cells, and CD8-GFP was found to be transcribed and translated in SM40K (Fig. 6a).

To further strengthen that GABA is regulated downstream of *GAD1* translation we applied the translation blocker cycloheximide. Because cycloheximide is cytotoxic, we had to minimize its incubation time. To this end, *elav::GAD1* cultures raised in SM40K for 3 days were either directly

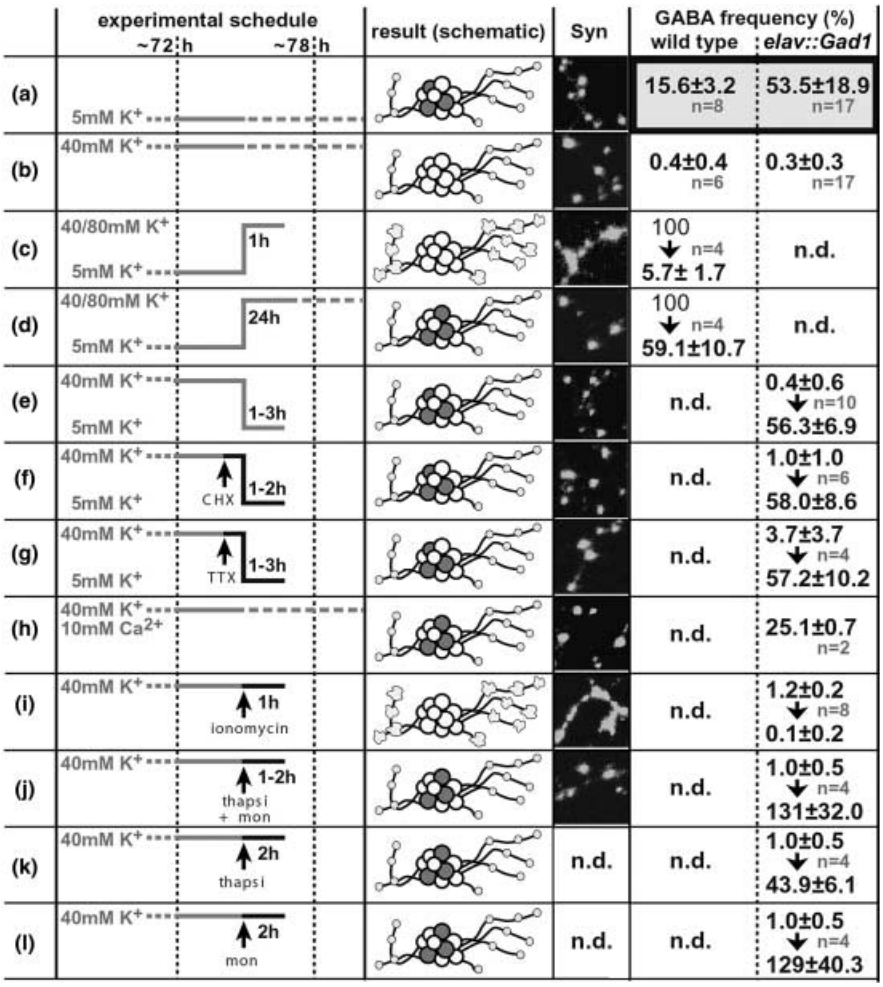

Fig. 4 Assaying GABA expression in primary cell cultures. Experimental schedule: a timeline is indicated, grey/black lines represent the period for which primary cell cultures were kept at high or low [K^+] (5 mM K^+, 40/80 mM K^+) in each particular experiment (a–l), arrows indicate timepoints from which pharmacological agents were applied (expressed also by colour change of line to black; CHX, 100–200 μg/mL cycloheximide; 5 μM ionomycin; mon, 5–10 μM monensin; thapsi, 5–10 μM thapsigargin; TTX, 3 μM tetrodotoxin). Result (schematic): representation of typical cell groups *in vitro* with cell bodies (large circles; GABA expression in dark) and their neurites (black lines) with pre-synaptic markers (small cicles, normal; small clouds; depleted). Syn: original images of pre-synaptic anti-Synapsin staining in respective cultures (diameter of each image 15 μm). GABA frequency (%): GABA frequencies of cultures obtained from wild-type (left) or *elav::GAD1* embryos (right). Values in (a) and (b) show direct percentages of GABA-positive cells (*n*, number of independent trials; for each trial 500–600 cells were counted), values in (c–l) are normalized to control cultures in SM5K [single SM5K data not shown, but comparable to those in grey box in (a)]. Values in (c–g) and (i–l) represent GABA frequencies before (above arrow) and after (below arrow) manipulation (n.d., non-determined).

stained (GABA < 1%) or shifted for 1–3 h to SM5K and stained thereafter. This short shift to SM5K was sufficient to elevate frequencies of GABA-expressing cells to about 30%. Parallel control cultures raised in SM5K throughout reached a GABA frequency of about 60%. Normalizing the data from shifted to control cultures shows that about half of the potentially GABA competent cells could be induced to express GABA through the shift to low [K^+] (shifted cultures 56.3 ± 6.9%, SM5K control cultures 100%; Fig. 4e). When we applied the translation inhibitor cycloheximide (100–200 μg/mL; see also Page and Orr-Weaver 1997) during this 2 h shift to SM5K, it was not able to suppress the upregulation of GABA staining (Fig. 4f). This supports our hypothesis that regulation of GABA production in our primary cell cultures via high [K^+] occurs downstream of *GAD1* translation, and is comparable to our observations in the developing embryonic CNS.

Suppression of GABA in chronically high [K^+] is independent of synaptic release

One explanation for reduced frequencies and intensities of GABA staining in high [K^+] media might be that cells are

permanently depolarized and thus depleted of GABA as has been demonstrated in vertebrate cell culture (e.g. Mellor *et al*. 1998; Maric *et al*. 2001). In order to test this possibility, cell cultures grown for 3 days in SM5K were shifted for 1 h to SM40K or SM80K. Compared to SM5K control cultures, GABA frequencies were severely reduced in the shifted cultures (5.7 ± 1.7% normalized to SM5K control culture; Fig. 4c). Parallel stainings for the pre-synaptic vesicle protein Synapsin, which normally localizes to small pre-synaptic spots along neuronal processes, suggested that this loss of GABA was due to synaptic depletion: within 1 h in SM40/80K Synapsin immunostaining extends into larger patches (Figa 4a vs. 4c). Similar changes of vesicular protein localization occur at *Drosophila* neuromuscular junctions upon vesicle depletion, because vesicular proteins become incorporated into the pre-synaptic membrane (Estes *et al*. 1996). Thus, cultured cells shifted from SM5K to SM40/80K for 1 h appear to release most of their GABA. However, if the period in SM40/80K is prolongued to 24 h, Synapsin staining once again appears punctuate, GABA immunoreactivity re-occurs with frequencies of 59.1 ± 10.7% (normalized to SM5K controls; Fig. 4d). In agreement with this observation, neurones chronically cultured in SM40K or SM80K throughout usually show punctuate staining of pre-synaptic vesicle proteins (but lack GABA; Fig. 4b). Hence, these neurones are not depleted but seem adapted to high $[K^+]$ conditions. In the light of continued depletion in vertebrate cell cultures (see above) this finding was surprising.

To gain insights into potential mechanisms underlying the observed GABA regulation, we analysed the state of SM40K cultured cells by performing whole-cell patch-clamp recordings on cultured neurones. Cells grown for 20–90 h in SM5K ($n = 131$) or SM40K ($n = 49$) were recorded in voltage clamp at −75 mV. A fraction of these (21% in SM5K; 35% in SM40K) displayed current fluctuations with kinetics reminiscent of post-synaptic currents (PSC; Fig. 5a; compare Lee and O'Dowd 1999). These events show a reversal potential at approximately 0 mV (not shown), are abolished by application of 1 μM α-bungarotoxin (Fig. 5b), and are therefore regarded as excitatory PSCs mediated by acetyl choline (in contrast, GABAergic events were not detectable for reasons not yet investigated). Hence, synaptic contacts are formed in both culture media. When PSC amplitudes were plotted they displayed essentially the same distribution in SM5K and SM40K and also frequencies were not significantly increased in high $[K^+]$ medium (Fig. 5d). In contrast, cells grown in SM5K and shifted to a bath solution containing 40 mM $[K^+]$ during the recording displayed a dramatic rise in PSCs (Fig. 5c). These observations are in agreement with our immunocytochemical observations, i.e. that immediately after a shift from SM5K to SM40K neurones are depleted (loss of GABA, changes of Synapsin pattern; Fig. 4c), but

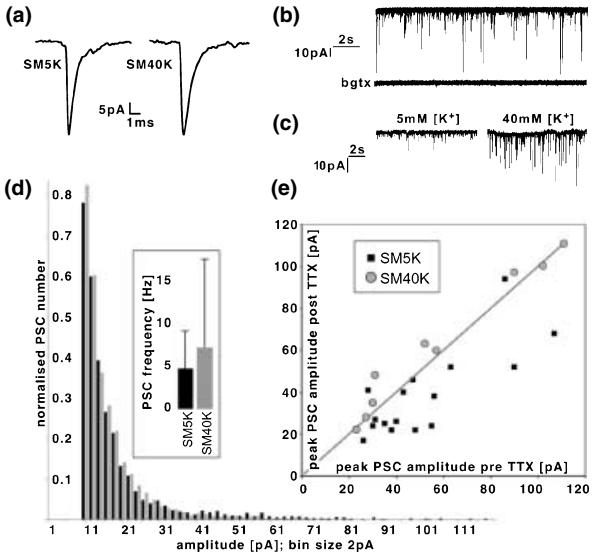

Fig. 5 Electrophysiological analyses of cultured neurones. (a) Typical trace of post-synaptic currents (PSC) recorded in SM5K (70 h *in vitro*; 30 individual events averaged) and SM40K (40 h *in vitro*; 14 events averaged). (b) Recording of one neurone before (top) and after (bottom) application of 1 μM α-bungarotoxin (bgtx; superimpositions of 20 sweeps of 800 ms, respectively). (c) Acute elevation of $[K^+]$ in the bath solution from 5 to 40 mM during the recording of neurones grown in SM5K causes dramatic increase in PSCs. (d) The distribution of PSC amplitudes from neurones (criteria: amplitude = 10 pA and frequency = 0.1 Hz) cultured in SM5K (black; $n = 27$) and SM40K (grey; $n = 17$) is comparable, as are their frequencies (inset). (e) Plotting peak amplitudes of PSCs (criteria: amplitude = 10 pA, frequency = 1 Hz) before tetrodotoxin application (pre-TTX) against amplitudes after application (post-TTX) reveals a tendency for amplitude reduction (data points below line) only in SM5K (black squares) but not in SM40K (grey circles) cultured neurones.

their synaptic activity appears adapted to high $[K^+]$ after a longer period (Fig. 5d).

To test whether spontaneous action potentials are generated in our cultures we characterized the effect of 1 μM tetrodotoxin (TTX; a blocker of voltage-gated Na^+ channels, e.g. O'Dowd 1995) on maximal PSC amplitudes (analysis restricted to cells with PSC frequencies = 1Hz). In SM40K, maximal PSC amplitudes were unaffected by TTX ($n = 9$; Fig. 5e, grey circles). In contrast, approximately half of the cells cultured in SM5K displayed reduced maximal PSC amplitudes upon TTX application ($n = 16$; Fig. 5e, black squares). Because action potentials elicit the largest PSCs in many preparations (e.g. Lee and O'Dowd 1999), this suggests that action potentials are generated at low frequencies in a fraction of cells grown in SM5K but are apparently absent from SM40K cultures. Thus, the adapted state of cells in SM40K seems to correlate with a reduced state of excitability. We tested whether genetic or pharmacological suppression of

excitation might have a direct impact on GABA regulation. However, neither TTX application to cell cultures (Fig. 4g) nor analysis of embryos lacking synaptic transmission (Table 1a) or lacking action potentials (Table 1b) showed any obvious effect on GABA expression.

Taken together, lack of GABA in cells chronically cultured in high [K^+] is not caused through depletion but occurs independently of neuronal release. Because cells in high [K^+] lack action potentials and display synaptic activity at frequencies comparable to cells kept in SM5K, the absence of GABA depletion seems to be due to reduced membrane currents in these cells. For example, reduction of Na^+ currents could be the cause for loss of action potentials (Fig. 5e), reduction of Ca^{2+} currents is a likely explanation for the adapted levels of synaptic activity (Fig. 5d; compare Lee and O'Dowd 1999). As GABA regulation in our cell cultures occurs downstream of *GAD1* translation (see above) but independent of release, we assume it to be mediated through regulation of the enzymatic activity of GAD1, as similarly suggested for developing *Drosophila* embryos (see above).

Increase of intracellular free [Ca^{2+}] is sufficient to induce GABA production

As suggested by the adapted levels of synaptic activity (Fig. 5d), chronic exposure to SM40K might influence Ca^{2+} currents. Because Ca^{2+} has enormous regulatory potential, and competence for GABA expression in embryos occurs at a time when Ca^{2+} currents can first be observed (Fig. 2a; Baines and Bate 1998), we wondered whether GAD1 activity might be controlled by Ca^{2+} signalling.

The possibilities of deleting *Drosophila* Ca^{2+} currents genetically are limited, as only a fraction of genes that encode for putative Ca^{2+} channels have been characterized sufficiently (Littleton and Ganetzky 2000). Two such genes are *Caα1A* (= *nightblind A*; N-type channel) and *Caα1D* (L-type channel). However, embryos with severe mutations of these genes tested alone or in double mutant constellations did not affect the GABA pattern at late stage 17 (Table 1c). Instead we focussed our experiments on cell cultures grown for 3 days in SM40K (Fig. 4). If extracellular [Ca^{2+}] was low (0.7 mM) in these cultures, almost no GABA was detectable (Fig. 4b), whereas 10 mM [Ca^{2+}] in SM40K was able to override the suppression of GABA (25.1 ± 0.7%, normalized to SM5K control cultures; Fig. 4h). One likely explanation for this effect is that elevation of extracellular [Ca^{2+}] might compensate for reduced freqencies of Ca^{2+} channel openings through increased Ca^{2+} influx per event. We reasoned that also pharmacological upregulation of intracellular free [Ca^{2+}] might be a feasible experimental approach to override the GABA blockade.

We tested ionomycin, thapsigargin and monensin, three commercial drugs which have been used previously in *Drosophila* to elevate intracellular free [Ca^{2+}] (e.g. Hardie 1996; Yagodin *et al*. 1999; MacPherson *et al*. 2001). These substances were applied for 1–2 h to cultures grown for 3 days in SM40K containing 0.7 mM [Ca^{2+}] (Figs 4i–l). Ionomycin is a (rather unspecific) ionophore causing Ca^{2+} influx across the cell membrane as well as release from non-acidic intracellular stores. Addition of 1–10 μM ionomycin did not induce GABA but depleted cells, as demonstrated by their complete loss of GABA and alterations of their Synapsin staining (Fig. 4i). It seems that the 0.7 mM [Ca^{2+}] in the medium (derived from the supplemented serum) is too high so that ionomycin-induced Ca^{2+} influx raised intracellular free [Ca^{2+}] above thresholds required for synaptic activation (see also Maric *et al*. 2001). We refrained from further reduction of extracellular [Ca^{2+}] via chelators as exposure of cells for more than 1 h to such unphysiological conditions is more likely to cause stress-related side-effects than deliver interpretable results. Instead we used thapsigargin and monensin which increase intracellular free [Ca^{2+}] by affecting intracellular Ca^{2+} stores. A total of 5–10 μM thapsigargin added to SM40K for 1–2 h raised the percentage of GABA-expressing cells to 43.9 ± 6.1% (Fig. 4k), and equal amounts of monensin increased numbers even more (129 ± 40.3%, normalized to SM5K control cultures; Fig. 4l). Both substances applied together reached similar values as monensin alone (Figs 4j and 6b′). This thapsigargin/monensin-mediated upregulation of GABA occurs downstream of translation as it was not suppressed by the translation blocker cycloheximide even when cycloheximide was applied 20 min before the [Ca^{2+}]-elevating drugs (data not shown). In a final experiment we tested whether inhibition of GABA production in the embryonic CNS could also be overridden by the same drugs. To this end, 10- to 11-hour-old wild-type or *elav::GAD1* embryos were dissected and cultured for 1–2 h in dissection buffer. Subsequent anti-GABA staining revealed hardly any staininig in control specimens (Figs 6c and e), but significant upregulation occurred if thapsigargin and monensin (20 μM each) were added during the culturing period (Figs 6d and f). Thus, commercial drugs known to raise intracellular free [Ca^{2+}] are sufficient to override GAD1 activity blockade of neurones in culture as well as in developing embryos.

In summary, we have shown that the timeline of GABA in the developing CNS is strictly controlled and our results strongly suggest that this regulation occurs at the level of GAD1 enzymatic activity. Because it is now possible to induce GABA during earlier periods of neurogenesis, a potential role of this GABA regulation in neurogenesis can be investigated.

Discussion

GABA in embryonic neurogenesis of *Drosophila*

Our studies were conducted in the context of a potential function of GABA during *Drosophila* neurogenesis. We describe, for the first time, the spatial and temporal pattern of GABA expression in the embryonic nerve cord of *Drosophila* (Figs 1–3). The numbers of GABA-expressing

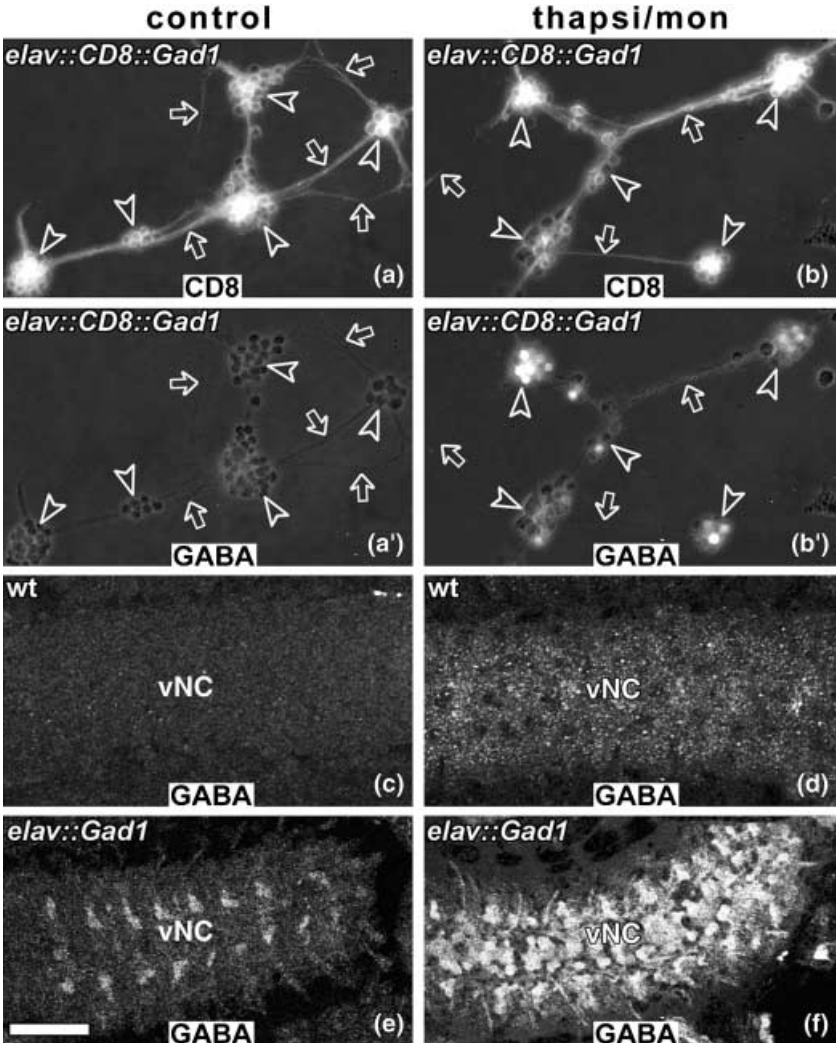

Fig. 6 Upregulation of GABA levels via drug treatment. (a–b′) Primary cell cultures (arrowheads, cell bodies; arrows, examples of neurites) obtained from embryos expressing CD8 and GAD1 pan-neuronally (*elav::CD8::GAD1*) grown in SM40K for 3 days show CD8 expression (a and b), but no GABA (a′), unless 5–10 μM thapsigargin and monensin were added for 2 h (bright staining in b′). (c–f) Ventral nerve cords (vNC; anterior left) of wild-type (wt; c and d) and of embryos with pan-neuronal expression of GAD1 (*elav::GAD1*; e and f) dissected at 10–11 h and cultured for 1–2 h in saline; levels of GABA are low in controls (c and e), but significantly upregulated if 20 μM thapsigargin and monensin were added to the saline (d and f). Scale bar 30 μm (a–b′) and 20 μm (c–f).

cells in the ventral nerve cord of *Drosophila* are very similar to those of other systems (our studies: approximately 20% in the nerve cord, approximately 15% in cell culture; other studies: 15% in another primary *Drosophila* cell culture, Lee and O'Dowd 1999; 5–25% in grasshopper CNS, reviewed in Burrows 1996; approximately 30% in primary cultures of rat post-natal visual cortex, Rutherford *et al*. 1997; 25–30% in monkey primary cortex, Hendry *et al*. 1987). However, the temporal pattern of GABA in *Drosophila* differs from other systems. In vertebrates, GABA appears during neurogenesis and seems to influence already early developmental processes like neural proliferation, migration and growth (see Introduction). However, our data suggest that such a role of GABA is not a common feature of developing nervous systems. We found that GABA expression in *Drosophila* becomes detectable hours after the onset of synaptogenesis, suggesting an early function for GABA to be unlikely. In agreement with this, we failed to detect obvious structural defects in *GAD1* mutant embryos lacking intrinsic GABA (unpublished data). However, these findings do not exclude a role of GABA during the refinement and plastic rearrangements of neuronal circuits.

The late appearance of GABA may be important for correct neuronal development. This is, in part, suggested by our findings that regulatory mechanisms efficiently suppress GAD1 activity during early development. We show the existence of such mechanisms by two parallel strategies, using the developing nerve cord and primary cell cultures. These mechanisms prevent even artificially elevated GAD1 levels from producing GABA (Fig. 3b′ vs. d′), demonstrating that they are not simply dose-dependent. Furthermore, their presence was demonstrated by the fact that they could be successfully overridden by manipulations of intracellular Ca^{2+} levels (Fig. 6). Interestingly, these inhibitory mechanisms seem to be tissue-specific as at 13 h targeted *GAD1* expression could induce GABA in muscles (N. Sanchez-Soriano, unpublished data) but not in the nervous system

(Fig. 3d′). Our hypothesis is that GABA has to be excluded from early neurogenesis, as it might lead to developmental aberrations at these stages due to its regulatory capabilities. In fact, parallel studies using misexpression of GABA receptors support this hypothesis (N. Sanchez-Soriano, unpublished data). An important outcome of the work presented here is that it is now feasible to test for such putative regulatory capabilities of GABA directly by overriding inhibition of GAD1 activity and analysing for potential effects on neurogenesis.

Drosophila as a model organism to study pathways regulating GAD activity

A second important outcome of our work is the establishment and successful use of the developing nerve cord and primary cell cultures as two parallel assay systems for the study of GAD regulation with pharmacology and with steadily advancing *Drosophila* genetics. Our data strongly suggest that the control mechanisms suppressing GABA act downstream of *GAD1* translation, and they are also likely to be independent of substrate supply, GABA-removing enzymatic activities and/or neuronal release. Furthermore, our data indicate that GAD1 activity can be upregulated by elevating intracellular free [Ca^{2+}]. The source (but not the amount) of Ca^{2+} seems irrelevant in cultured cells: GABA upregulation could be achieved through elevation of extracellular [Ca^{2+}] but also through very specific intracellular store release via thapsigargin and monensin (Figs 4h and j–l). The same drugs elevated GABA levels also in developing embryos (Fig. 6). Because intrinsic GABA upregulation in developing embryos correlates with the time when voltage-dependent Ca^{2+} currents occur (Fig. 2a), influx of extracellular Ca^{2+} might be the key regulator under normal conditions. Analyses of (unfortunately not yet available) mutations affecting Ca^{2+} channels would have to prove this hypothesis (see Table 1c).

In this context, our findings in *Drosophila* might link directly to work from vertebrates showing that enzymatic activity of GAD is regulated by free Ca^{2+} (e.g. Bao *et al*. 1994; Erecinska *et al*. 1996). This regulation seems to be mediated by Ca^{2+}-dependent phosphatases and kinases altering the phosphorylation state of GAD (Bao *et al*. 1994, 1995). Regulation of GAD activity via Ca^{2+} binding proteins has also been reported in yeast and plants (Coleman *et al*. 2001 and references therein). Thus, it seems reasonable to speculate that control of GAD activity by Ca^{2+}-signalling pathways in different species might share common regulatory components. Therefore, insights gained with the help of the steadily improving genetics in *Drosophila* (FlyBase 1999) might help to advance a general understanding of the molecular mechanisms underlying the regulation of GAD activity.

A potential application of such a knowledge might be in the context of neurological diseases like epilepsy. Thus, ketogenic diet as a cure for epilepsy has been shown to work via upregulation of GAD1 enzymatic activity (Erecinska *et al*. 1996 and references therein). It seems feasible to use mechanisms controlling GAD activity as potential targets for antiepileptic drug design. This potential has not been extensively studied, most likely due to the fact that underlying regulatory pathways are poorly understood (reviewed in Böhme and Lüddens 2001). *Drosophila* might prove to be a suitable model to improve this knowledge.

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