G protein αq gene expression plays a role in alcohol tolerance in Drosophila melanogaster.

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Title

G protein alpha-q gene expression plays a role in alcohol tolerance in *Drosophila melanogaster.*

Authors

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Abstract

Ethanol is a psychoactive substance causing both short and long-term behavioural changes, in humans and animal models. We have used the fruit fly *Drosophila melanogaster* to investigate the effect of ethanol exposure on the expression of the Gαq protein subunit. Repetitive exposure to ethanol causes a reduction in sensitivity (tolerance) to ethanol which we have measured as the time for 50% of a set of flies to become sedated after exposure to ethanol (ST50). We demonstrate that the same treatment that induces an increase in ST50 over consecutive days (tolerance) also causes a decrease of Gαq protein subunit expression both at the mRNA and protein level. To identify whether there may be a causal relationship between these two outcomes, we have developed strains of flies in which Gαq mRNA expression is suppressed in a time and tissue specific manner. In these flies, the sensitivity to ethanol and the development of tolerance is altered. This work further supports the value of *Drosophila* as a model to dissect the molecular mechanisms of the behavioural response to alcohol and identifies G proteins as potentially important regulatory targets for alcohol use disorders.

Introduction

Ethanol, the alcohol most commonly found in fermented beverages, causes both acute and chronic effects on human and animal behaviour. The acute effects are known to be mediated via alteration of the activity of a number of central nervous system receptors and voltage-gated ion channels (Camarini and Pautassi, 2016). In contrast, long-term ethanol-induced changes in behaviour, which include tolerance, craving, withdrawal and relapse are regulated by less-well understood mechanisms. Prior work has indicated the involvement, among other molecules, of G-protein coupled receptors (GPCR). This includes dopamine-, serotonin-, $GABA_B$- opiate-, and other peptide receptors (Lovinger and Roberto, 2013). Additionally G-protein coupled receptors have been shown to facilitate GABA release following ethanol stimulation (Kelm et al., 2011). However, less attention has been given to the role of the G-proteins associated with these receptors. GPCR are stimulated by extracellular ligands and transduce the signal by activating their associated G-proteins.

G-proteins are heterotrimeric complexes composed of alpha, beta and gamma subunits of which several subtypes are encoded by the genomes of individual animal species (Milligan and Kostenis, 2006). Different G-protein subtypes elicit individual cellular signalling events by activating or inhibiting a variety of specific enzymes that further transduce the signal to other cellular systems (Syrovatkina et al., 2016). Each GPCR tends to associate with specific trimers of G-protein subunits, however promiscuity of GPCR and G-protein interaction has been reported as a result of changes in G-protein gene expression for some but not all receptors (Kostenis et al., 2005; Camarini and Pautassi, 2016). Recent analysis of G-protein subunits and GPCR using cryo-microscopy has revealed both similarities and differences in the interaction between different GPCR and G-proteins (Capper and Wacker, 2018) which supports the possibility of receptors associating with different G proteins.

The study of the molecular effects of ethanol have been greatly facilitated by the use of animal models (Barkley-Levenson and Crabbe, 2012). The fruit fly *Drosophila melanogaster* offers several advantages over mammalian models due to simple behaviours, short generation time, and amenability to genetic studies (Kaun et al., 2012). When repeatedly exposed to sedating doses of ethanol, *Drosophila* display tolerance measurable as a delayed onset of sedation in later ethanol exposures compared to the first exposure (Morozova et al., 2006; Sandhu et al., 2015). These behavioural changes are likely to depend on gene expression changes. However, the specific genes involved, and their temporal sequence of activation or inactivation is not known. In *Drosophila*, RNA microarrays have successfully been used to identify several classes of genes whose expression is affected by alcohol treatment (Kong et al., 2010). An alternative approach is to focus on candidate genes based on their known involvement in the processes being investigated. In this study, we have hypothesised that changes in G-protein expression play a role in alcohol induced tolerance in *Drosophila* as such change in expression could result in changes in the association of the G proteins with receptors and thus lead to alteration in cellular signalling in response to drugs (tolerance) or in their absence (craving). Changes in G-proteins gene expression induced by psychoactive drugs have been previously documented in mammalian systems (Kaewsuk et al., 2001; Kitanaka et al., 2008; Zelek-Molik et al., 2012), but to our knowledge this has not been documented in *Drosophila* for alcohol induced behaviours.

Following an initial screening of *Drosophila* G-proteins (Supplementary Table 1), in this work we have investigated the effect of alcohol on the expression of Gαq and we demonstrate a correlation between downregulation of this subunit and the onset of tolerance.

Results

Development of ethanol tolerance in wild-type *Drosophila*

Drosophila wild type Canton-S 1-3 days old males exposed to ethanol vapours responded by reducing their locomotion followed by sedation. Sedation was determined by observing the flies every minute and recording the number of flies that were not able to recover to an upright position after being startled. The time at which 50% of the flies in the same exposure chamber were sedated was recorded as the ST50 for that group of eight flies. Flies were exposed to the same ethanol treatment for three consecutive days at 24 hr intervals and, as expected, a higher ST50 was observed on the second and third day when compared to the first day of exposure indicating that the flies were less responsive to the sedating effect of ethanol and thus more 'tolerant' (Figure 1). A control experiment where ST50 was measured one or three days after selection and receiving the same handling as chronically treated flies but with no alcohol exposure other than measuring one ST50 showed no age induced development of tolerance (results not shown).

Alcohol effect on Gαq expression in wild-type *Drosophila*

RNA was extracted from the heads of *Drosophila* sacrificed at different time points during tolerance development: naïve untreated flies (control); 1 hour after the first ethanol exposure (acute response); 24 hr after the second ethanol exposure (basal level in 'chronically' treated flies); one hour after the third exposure (acute response in 'chronically' treated flies). A significant decrease in Gαq mRNA expression was observed in basal level and in the acute response of 'chronically' treated flies (Figure 2). We use the term 'chronically treated' to emphasise the shift in response as compared to the first treatment. To confirm that the change in mRNA expression had an effect on protein levels, western blots were carried out with a primary antibody that recognises *Drosophila* Gαq protein. A significant reduction of Gαq protein was observed in chronically treated flies (Figure 3).

Effect of induced down-regulation of Gαq on tolerance development

In order to determine whether there is a causal relationship between the observed concurrent development of tolerance and down regulation of Gαq, knockdown of Gαq expression was induced via Gal4-UAS RNAi (Brand and Perrimon, 1993). To avoid that the reduced expression of Gαq affected the normal development of the flies, the induction of the inhibitory RNA (RNAi) was regulated in the flies by the temperature-sensitive Gal4 suppressor tubulin-Gal80^{ts} (McGuire et al., 2003). Gal80^{ts} is inactivated at temperatures of 25°C and above, thus flies maintained at 18°C would not express the Gαq RNAi and express normal level of Gαq while at 25°and above the expression of Gαq is suppressed by Gαq RNAi. We present here the data for two different lines that we have developed through crossings: one in which Gal4 is driven by the promoter of ubiquitously expressed tubulin (Tub-Gal4-Gal80ts-siRNAGαq) and one driven by the promoter of the neuronally expressed elav (Elav-Gal4-Gal80ts-siRNAGαq) both constructs also expressing tubulin-Gal80^{ts}. In both fly lines we confirmed a significant reduction of Gαq mRNA expression at the higher temperature as compared to 18°C (Figure 4). It should be noted that we carried out the RNAi induction at 30°C and 25°C for the tubulin and elav constructs respectively, because we had observed that the shift to 30°C (but not to 25°C) moderately affected the same-background control line of the elav construct (result at 30°C for elav construct not shown). The Tub-Gal4-Gal80ts-siRNAGαq and the Elav-Gal4-Gal80ts-siRNAGαq were both subjected to the tolerance protocol (described above) at 18°C and 25°C/30°C and the respective ST50 were measured (Figure 5). At 18°C all flies demonstrated an increase of ST50 (tolerance) over the three ethanol exposures. At 25°C/30°C both constructs with siRNAGαq flies demonstrated a higher ST50 on the first ethanol exposure than at 18°C and did not demonstrate an increase in ST50 (no tolerance) over the next two ethanol exposures. As the genetic background can in some cases affect ethanol induced behaviour (Chan et al., 2014) we measured tolerance development in flies resulting from crosses of w^{1118};tub−Gal80ts ; tub−Gal4/TM6c-Sb, and w^{1118}, elav-GAL4, mw+ ; tub-GAL80ts, mw+ ; + and a fly line with the same background of the siRNAGαq line but not containing siRNAGαq. We observed that there was no difference in the ST50 at day one between 18°C and 25/30°C, and that normal tolerance developed over 3 days of ethanol exposure at both 18°C and 25/30°C. (Supplementary Figure 2) .

Discussion (1500 max)

In this work, *Drosophila* was confirmed to be a useful model for studying alcohol induced behaviours as has been amply demonstrated in other studies (Kaun et al., 2012; Robinson et al., 2013). Previous *Drosophila* work had identified a number of GPCR that are involved in the response to alcohol including the Dopamine/Ecdysteroid Receptor (Petruccelli et al., 2016), Neuropeptide F receptor (Wen et al., 2005) putative opioid receptors (Koyyada et al., 2018) and GABA$_B$ receptor (Ranson et al., 2019). An earlier extensive review of mammalian studies of the effect of psychostimulants on G-protein expression (Kitanaka et al., 2008) highlighted that only limited work had been focussed on ethanol-induced changes in any animal models with only one study reporting a reduction of G-protein b1 in rat hippocampus (Saito et al., 2002). A more recent microarray study (Kong et al., 2010) focusing on the effect of acute ethanol exposure in *Drosophila* did not identify any G-proteins being significantly affected. This matches with our observations of a lack of significant expression change following acute exposure as opposed to chronic exposure which does cause a significant change compared to untreated flies. To our knowledge, this is the first report specifically targeting ethanol-induced G-protein changes and the first to be carried out in *Drosophila*.

This study has demonstrated that chronic exposure to ethanol causes a reduction of Gαq expression in *Drosophila* heads. We have confirmed that this statistically significant decrease occurs both at the mRNA and protein level. Additionally, the results strongly suggest that this altered Gαq expression has a functional significance, as flies in which Gαq expression was downregulated via Gαq-RNAi show an altered behaviour in the development of tolerance to ethanol. Given that chronic ethanol exposure induces a Gαq reduction and a reduction in the sensitivity to ethanol (increase in ST50), and given that RNA reduction of Gαq causes an increase in ST50 similar to chronic ethanol exposure, it is reasonable to hypothesise that Gαq is involved in the reduction of sensitivity to ethanol following chronic ethanol exposure. We have demonstrated this effect both in ubiquitously expressed Gαq down regulation (tubulin promoter driven) and in neuron specific down regulation (elav promoter driven). Gαq is known to have important neuronal function in the *Drosophila* brain (Himmelreich et al., 2017) and thus it might have been expected that neuronally restricted downregulation to be more effective. Indeed in the elav construct, a 50% reduction of Gαq mRNA had very similar effect in terms of ST50 change to a 81% mRNA reduction of the tubulin construct. The mechanism by which the change in Gαq expression and its effect on tolerance occurs remains to be elucidated in terms of how the Gαq gene is regulated and how the change of expression is associated with tolerance. The slo-K$^+$ channel (homologous to the mammalian BK channel) has been implicated in the formation of rapid tolerance to ethanol (Ghezzi et al., 2004) and it would be of interest to determine if there functional link between slo-K$^+$ and Gαq with respect to alcohol tolerance.

The Gαq *Drosophila* subunit is known to signal via the phospholipase C pathway which leads to activation of Protein Kinase C (Litosch, 2016). Interestingly, a deficiency of protein kinase C has been associated with desensitisation to alcohol in *Drosophila* (Chen et al., 2010). This would be consistent with our finding that ethanol induced reduction of Gαq is

associated with reduced sensitivity for alcohol. It may also be relevant that Slo-K$^+$ activity is affected by PKC phosphorylation (Contreras et al., 2013). Recent findings have depicted a more complex picture of G-protein signalling, which includes multiple targets for Gαq (Litosch, 2016); multiple isoforms of phospholipase C, and the role of G-protein regulating proteins (McCudden et al., 2005). Full understanding of the role of Gαq in ethanol-induced behaviour will require understanding the role of multiple physiological functions. While in this study we have specifically focussed on the relation between Gαq and ethanol-induced tolerance, Gαq due to its wide distribution and association with multiple receptors is associated with several other functions. Indeed Gαq mutants have been shown to have altered olfactory expression (Kain et al., 2008) and axonal pathfinding (Ratnaparkhi et al., 2002). However, the advantage of measuring ST50, as we did in this study, is that the time to sedation is directly related to the exposure to ethanol and is not affected by other functions such as olfaction, memory or directional movement.
It also needs to be established to what extent the change in gene expression for Gαq is specific to alcohol consumption as compared to other psychoactive substances. In cocaine treated rats, a significant increase of Gαq was observed in the amygdala and paraventricular nucleus membrane fraction two days after withdrawal with no change in the frontal cortex or in the cytosolic fraction of any of the brain regions (Carrasco et al., 2003).

The Gαq subunit is probably not the only subunit whose expression is affected by alcohol. Indeed, we have preliminary data for changes in other alpha and beta subunits, but these observations require confirmation by further genetic studies.

In summary, this work provides evidence that Gαq expression is affected by chronic alcohol exposure and that this change is likely to be involved in the development of tolerance. Further work analysing different G-proteins subunits and other effectors of G-protein signalling needs to be carried to fully elucidate the mechanism of tolerance to alcohol and other psychoactive drugs in *Drosophila* and mammalian species.

Methods

Fly stock and maintenance

Canton-S wild-type flies, siRNA line for Gαq specific knock down (Stock number 36775), and a line with same background of the siRNA line (stock number 36303) were obtained from Bloomington stock centre USA. *Drosophila* lines w^{1118};tub−Gal80ts ; tub−Gal4/TM6c-Sb (kindly donated by Professor Joerg Albert, UCL, UK) and w^{1118}, elav-GAL4, mw+ ; tub-GAL80ts, mw+ ; + (kindly donated by Dr Colin McClure, Imperial College London, UK) . Fly lines with temperature inducible expression of Gαq RNAi were developed by crossing Gαq RNAi virgin females with male w^{1118}/Y ;tub−Gal80ts ; tub−Gal4/TM6c-Sb or w^{1118}, elav-GAL4, mw+ ; tub-GAL80ts, mw+ ; + flies. Male offspring were selected based on lack of the dominant stubble marker for the Tub-Gal4 driver, while flies with the elav-Gal4 driver did not need selection because the parent fly was homologous for elav-Gal4 and tub-GAL8ts. The flies resulting from these crosses will be referred to as Tub-Gal4-Gal80ts-siRNAGαq and Elav-Gal4-Gal80ts-siRNAGαq where the former is expected to express siRNAGαq ubiquitously while the latter only in neurons; the expression siRNAGαq will be repressed by Gal80ts in both lines at 18°C. All flies were grown on Ready-mix *Drosophila* dried food prepared with water in equal amounts (Philip Harris Education, UK), and routinely incubated at 25^{0}C, 60% relative humidity in a 12 h light/dark cycle incubator. Temperature sensitive mutant flies were reared at either 18^{0}C (control) or 25-30^0 C (experimental conditions: 25°C was used for Elav-Gal4-Gal80ts-siRNAGαq and 30°C for Tub-Gal4-Gal80ts-siRNAGαq).

Behaviour assay

Ethanol sedation and tolerance were measured using the procedure adapted from Maples and Rothenfluh (2011). Male flies were separated in groups of 8 using light CO_2 sedation and allowed to recover in a tube with food for 24 h. Flies were selected from actively growing colonies that were cleared 72 h earlier and may have thus contained different ratio of fly ages. Flies were transferred to a 25x95mm transparent plastic vial in between two cotton plugs. One cotton plug at the base of the vial served as a stable surface to observe the flies and the other cotton plug was used to cap the vial and deliver the ethanol. 500µl of 100% ethanol was added to the side of the cotton plug facing the flies. Sedation was observed manually as ST50, which is the time in minutes it takes for 50% of the flies in a sample vial to become sedated. Sedation was defined as the lack of movement or the inability to self-right for 3 sec after being startled to the bottom of the tube. The 3 sec observation has been optimised to reduce observer bias. It is extremely rare for flies that have been stationary for 3 sec to right themselves while still being in the ethanol chamber. Flies were exposed to ethanol for 3 consecutive days, once a day with 24h in between exposures. Experiment series were repeated on different days with different generations of flies.

Sequence analysis and primer design

The DNA sequence for Gαq protein genes in the Drosophila genome were obtained from Fly Base (www.flybase.org) and/or National Centre for Biotechnology Information Databases (Gq: CG17759) and aligned using CLUSTALW2 (www.ebi.ac.uk/tools/ms/clustalW2), a free online tool through the European Bioinformatics Institute (EBI). Primer pairs were designed using National Centre for Biotechnology Information to span intron regions or exon-exon junction in order to avoid amplifying contaminating genomic DNA. A pair of primers was designed for the G-protein gene and the sequences were verified by a BLAST search to check for specificity to the Gαq protein coding regions. The following primers were designed: (Gq gene) Gq fwd: 5'-CAGCAGCACGCGAAAGCGTC-3' and Gq rev: 5'-GTCCCGGCGCAACTGCTTCT-3'. The housekeeping gene/internal control, (β-actin) β-actin fwd: 5'- GCGTCGGTCAATTCAATCTT -3' and β-actin rev: 5'AAGCTGCAACCTCTTCGTCA -3' were selected from a previous study (Ponton et al., 2011).

Real time RT-PCR

Flies (sets of eight) were snap frozen in liquid nitrogen (30 sec), the heads were isolated by 2 min vortex decapitation and collected under a dissecting microscope. Heads were homogenised with disposable tissue homogenisers for 10 sec in Qiagen RNEasy Plus RLT buffer in sterile 1.5ml Eppendorf tubes according to the manufacturer's instructions. RNA was quantified spectrophotometrically (Nanodrop Technologies, Wilmington, US). RNA was amplified and quantified with the one-step RT PCR quantification kit from PCR Biosystems (UK) on a Stratagene Mx3000pTM Real-Time PCR System (Stratagene, US) according to the manufacturer's instructions. Each reaction mixture contained the following: 5 ng of RNA, 400 nM of forward and reverse primer, made up to 20 µL with the kit reagents, in a 96-well plate (Thermo Scientific, UK). qRT-PCR was performed under the following sequential conditions according to manufacturer's protocol: cDNA synthesis at 45^{0}C for 10 min, polymerase activation at 95^{0}C for 2 min, initial denaturation at 95^{0}C for 5 sec, followed by 40 cycles of denaturation at 95^{0}C for 5 sec and annealing/extension at 60^{0}C for 20 sec. In each experiment, a melting curve cycle was performed according to the manufacturer's programme to check the melting temperature of the products produced to ensure the product was of the expected size and not the result of primer-dimers. mRNA level was quantified using the comparative method ($2^{-\Delta\Delta Ct}$), (Schmittgen and Livak, 2008), where $2^{-\Delta\Delta Ct}$ equals the normalised threshold cycle (DCt) of G-protein genes in treated flies minus the DCt of the same gene in naïve flies (control) and normalised to the internal control β-actin. The efficiency of the primers was measured and were found to be comparable to satisfy the recommendations of (Pfaffl, 2001). RT values ranged between 21- 23 and 20-21 for Gq and actin respectively.

Western Blot

The following antibodies were used: rabbit polyclonal anti-Gαq/11 at a dilution of 1:250 (Santa Cruz Biotech. Inc. US), mouse monoclonal anti-Gαq/11 (sc-136181, Santa Cruz Biotech. Inc. US) at a dilution of 1:250; anti-actin at a dilution of 1:3000 (St John's Laboratory, UK); horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG secondary antibody (Cayman Chemical Company, UK), at dilution 1:10000. Sets of 15 flies heads (males) were homogenised in Laemmli buffer in sterile and ice cold 1.5 mL eppendorf tubes. Samples were then cooled in ice for 1 min and heated for 5 min at 95^{0}C. After further cooling on ice for 1 min, the samples were centrifuged at top speed for 1 min and resolved on 10% SDS gel (ProtoGel- National diagnostics). Blots were transferred on to 0.2 µm pore sized PVDF membranes (Biorad) using *Trans-blot turbo.* The membrane was incubated with the primary antibodies for 18 h at 4°C, washed three times with TBS buffer 0.05% Tween20, incubated with secondary antibodies for 1 h at room temperature developed with Biorad chemiluminescence reagents and visualised using *Biorad ChemiDoc* imager. The same membranes were prepared for reprobing by incubation with 0.2M NaOH for 5 min followed by three washes in water. Membranes were incubated with the anti-actin antibodies for 2 h at room temperature and further processed as described above. Molecular weight markers were Magic marker XP from Fisher Scientific UK.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7. Statistical tests are indicated in the figure legends. Western blots were analysed using Image J. Error bars represent mean ± standard error of the mean (SEM) or standard deviation.

Authors contribution statements

BA carried out most of the experimental work described in the manuscript, OU had carried out the initial development work that lead to the findings here described, RK provided expertise in fly genetics, DCR contributed to the final experiments. AT provided molecular biology expertise and advice during the project, OC co-supervised the project, SOC directed the project. All authors contributed to the completion of the manuscript and reviewed it before submission.

Competing interest statement

None of the authors have any competing interest in the publication of the data described.

Data Availability statement

All the data presented in this manuscript and any supporting data will be made available

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Bibliography

Barkley-Levenson, A.M., Crabbe, J.C., 2012. Bridging Animal and Human Models. Alcohol Res. Curr. Rev. 34, 325–335.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Dev. Camb. Engl. 118, 401–415.

Camarini, R., Pautassi, R.M., 2016. Behavioral sensitization to ethanol: Neural basis and factors that influence its acquisition and expression. Brain Res. Bull. 125, 53–78. https://doi.org/10.1016/j.brainresbull.2016.04.006

Capper, M.J., Wacker, D., 2018. How the ubiquitous GPCR receptor family selectively activates signalling pathways. Nature 558, 529. https://doi.org/10.1038/d41586-018-05503-4

Carrasco, G.A., Zhang, Y., Damjanoska, K.J., D'Souza, D.N., Garcia, F., Battaglia, G., Muma, N.A., Van de Kar, L.D., 2003. A region-specific increase in Galphaq and Galpha11 proteins in brains of rats during cocaine withdrawal. J. Pharmacol. Exp. Ther. 307, 1012–1019. https://doi.org/10.1124/jpet.103.056978

Chan, R.F., Lewellyn, L., DeLoyht, J.M., Sennett, K., Coffman, S., Hewitt, M., Bettinger, J.C., Warrick, J.M., Grotewiel, M., 2014. Contrasting influences of Drosophila white/mini-white on ethanol sensitivity in two different behavioral assays. Alcohol. Clin. Exp. Res. 38, 1582–1593. https://doi.org/10.1111/acer.12421

Chen, J., Zhang, Y., Shen, P., 2010. Protein kinase C deficiency-induced alcohol insensitivity and underlying cellular targets in Drosophila. Neuroscience 166, 34–39. https://doi.org/10.1016/j.neuroscience.2009.12.009

Contreras, G.F., Castillo, K., Enrique, N., Carrasquel-Ursulaez, W., Castillo, J.P., Milesi, V., Neely, A., Alvarez, O., Ferreira, G., González, C., Latorre, R., 2013. A BK (Slo1) channel journey from molecule to physiology. Channels 7, 442–458. https://doi.org/10.4161/chan.26242

Ghezzi, A., Al-Hasan, Y.M., Larios, L.E., Bohm, R.A., Atkinson, N.S., 2004. slo K+ channel gene regulation mediates rapid drug tolerance. Proc. Natl. Acad. Sci. U. S. A. 101, 17276–17281. https://doi.org/10.1073/pnas.0405584101

Himmelreich, S., Masuho, I., Berry, J.A., MacMullen, C., Skamangas, N.K., Martemyanov, K.A., Davis, R.L., 2017. Dopamine Receptor DAMB Signals via Gq to Mediate Forgetting in Drosophila. Cell Rep. 21, 2074–2081. https://doi.org/10.1016/j.celrep.2017.10.108

Kaewsuk, S., Hutamekalin, P., Ketterman, A.J., Khotchabhakdi, N., Govitrapong, P., Casalotti, S.O., 2001. Morphine induces short-lived changes in G-protein gene expression in rat prefrontal cortex. Eur. J. Pharmacol. 411, 11–16.

Kain, P., Chakraborty, T.S., Sundaram, S., Siddiqi, O., Rodrigues, V., Hasan, G., 2008.

Reduced odor responses from antennal neurons of G(q)alpha, phospholipase Cbeta, and rdgA mutants in Drosophila support a role for a phospholipid intermediate in insect olfactory transduction. J. Neurosci. Off. J. Soc. Neurosci. 28, 4745–4755. https://doi.org/10.1523/JNEUROSCI.5306-07.2008

Kaun, K.R., Devineni, A.V., Heberlein, U., 2012. Drosophila melanogaster as a model to study drug addiction. Hum. Genet. 131, 959–975. https://doi.org/10.1007/s00439-012-1146-6

Kelm, M.K., Criswell, H.E., Breese, G.R., 2011. Ethanol-enhanced GABA release: A focus on G protein-coupled receptors. Brain Res. Rev. 65, 113–123. https://doi.org/10.1016/j.brainresrev.2010.09.003

Kitanaka, N., Kitanaka, J., Hall, F.S., Tatsuta, T., Morita, Y., Takemura, M., Wang, X.-B., Uhl, G.R., 2008. Alterations in the levels of heterotrimeric G protein subunits induced by psychostimulants, opiates, barbiturates, and ethanol: Implications for drug dependence, tolerance, and withdrawal. Synap. N. Y. N 62, 689–699. https://doi.org/10.1002/syn.20543

Kong, E.C., Allouche, L., Chapot, P.A., Vranizan, K., Moore, M.S., Heberlein, U., Wolf, F.W., 2010. Ethanol-Regulated Genes That Contribute to Ethanol Sensitivity and Rapid Tolerance in Drosophila. Alcohol. Clin. Exp. Res. 34, 302–316. https://doi.org/10.1111/j.1530-0277.2009.01093.x

Kostenis, E., Waelbroeck, M., Milligan, G., 2005. Techniques: promiscuous Galpha proteins in basic research and drug discovery. Trends Pharmacol. Sci. 26, 595–602. https://doi.org/10.1016/j.tips.2005.09.007

Koyyada, R., Latchooman, N., Jonaitis, J., Ayoub, S.S., Corcoran, O., Casalotti, S.O., 2018. Naltrexone Reverses Ethanol Preference and Protein Kinase C Activation in Drosophila melanogaster. Front. Physiol. 9, 175. https://doi.org/10.3389/fphys.2018.00175

Litosch, I., 2016. Decoding Gαq signaling. Life Sci. 152, 99–106. https://doi.org/10.1016/j.lfs.2016.03.037

Lovinger, D.M., Roberto, M., 2013. Synaptic Effects Induced by Alcohol. Curr. Top. Behav. Neurosci. 13, 31–86. https://doi.org/10.1007/7854_2011_143

Maples, T., Rothenfluh, A., 2011. A simple way to measure ethanol sensitivity in flies. J. Vis. Exp. JoVE. https://doi.org/10.3791/2541

McCudden, C.R., Hains, M.D., Kimple, R.J., Siderovski, D.P., Willard, F.S., 2005. G-protein signaling: back to the future. Cell. Mol. Life Sci. CMLS 62, 551–577. https://doi.org/10.1007/s00018-004-4462-3

McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., Davis, R.L., 2003. Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302, 1765–1768. https://doi.org/10.1126/science.1089035

Milligan, G., Kostenis, E., 2006. Heterotrimeric G-proteins: a short history. Br. J. Pharmacol. 147, S46–S55. https://doi.org/10.1038/sj.bjp.0706405

Morozova, T.V., Anholt, R.R.H., Mackay, T.F.C., 2006. Transcriptional response to alcohol exposure in Drosophila melanogaster. Genome Biol. 7, R95. https://doi.org/10.1186/gb-2006-7-10-r95

Petruccelli, E., Li, Q., Rao, Y., Kitamoto, T., 2016. The Unique Dopamine/Ecdysteroid Receptor Modulates Ethanol-Induced Sedation in Drosophila. J. Neurosci. Off. J. Soc. Neurosci. 36, 4647–4657. https://doi.org/10.1523/JNEUROSCI.3774-15.2016

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Ponton, F., Chapuis, M.-P., Pernice, M., Sword, G.A., Simpson, S.J., 2011. Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster. J. Insect Physiol. 57, 840–850. https://doi.org/10.1016/j.jinsphys.2011.03.014

Ranson, D.C., Ayoub, S.S., Corcoran, O., Casalotti, S.O., 2019. Pharmacological targeting of the GABAB receptor alters Drosophila's behavioural responses to alcohol. Addict. Biol. https://doi.org/10.1111/adb.12725

Ratnaparkhi, A., Banerjee, S., Hasan, G., 2002. Altered levels of Gq activity modulate axonal pathfinding in Drosophila. J. Neurosci. Off. J. Soc. Neurosci. 22, 4499–4508. https://doi.org/20026385

Robinson, B.G., Khurana, S., Atkinson, N.S., 2013. Drosophila larvae as a model to study physiological alcohol dependence. Commun. Integr. Biol. 6, e23501. https://doi.org/10.4161/cib.23501

Saito, M., Smiley, J., Toth, R., Vadasz, C., 2002. Microarray analysis of gene expression in rat hippocampus after chronic ethanol treatment. Neurochem. Res. 27, 1221–1229.

Sandhu, S., Kollah, A.P., Lewellyn, L., Chan, R.F., Grotewiel, M., 2015. An Inexpensive, Scalable Behavioral Assay for Measuring Ethanol Sedation Sensitivity and Rapid Tolerance in Drosophila. J. Vis. Exp. JoVE. https://doi.org/10.3791/52676

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.

Syrovatkina, V., Alegre, K.O., Dey, R., Huang, X.-Y., 2016. Regulation, Signaling and Physiological Functions of G-proteins. J. Mol. Biol. 428, 3850–3868. https://doi.org/10.1016/j.jmb.2016.08.002

Wen, T., Parrish, C.A., Xu, D., Wu, Q., Shen, P., 2005. Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. Proc. Natl. Acad. Sci. U. S. A. 102, 2141–2146. https://doi.org/10.1073/pnas.0406814102

Zelek-Molik, A., Bielawski, A., Kreiner, G., Popik, P., Vetulani, J., Nalepa, I., 2012. Morphine-induced place preference affects mRNA expression of G protein α subunits in rat brain. Pharmacol. Rep. PR 64, 546–557.

Figures and legends

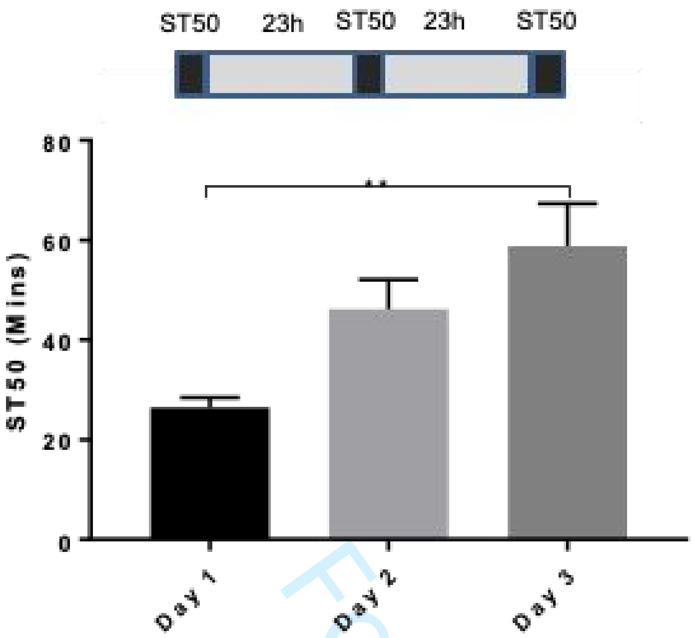


Figure 1 Sedation time (ST50) in wild type *Drosophila*. Groups of 8 male flies were exposed to 100% EtOH vapours and ST50 (time until 50% of the flies were sedated) was recorded. The timeline indicates that the three ST50 assays were carried out on three consecutive day with 24 h intervals. Horizontal bar indicates significant difference over three days, one way ANOVA $^{**}p < 0.01$, n=6 independent experiments on separate days. Error bars = SEM.

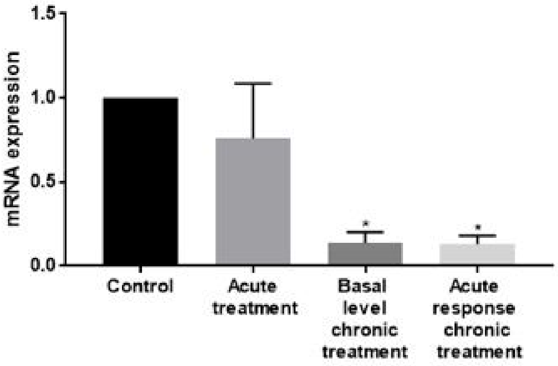


Figure 2 qRT-PCR of Gαq mRNA in wild type *Drosophila*. mRNA levels of the Gaq subunit were quantified from the heads of control and ethanol-exposed wild type flies by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method and normalized to an internal control β-actin. Flies (sets of 8) were exposed to ethanol for 30 min at 24 h intervals for up to three days, and were sacrificed either before ethanol exposure (Control), 1 h after the end of the first ethanol exposure (Acute treatment), 24 h after the second ethanol exposure (Basal level chronic treatment) or 1 h after the end of the third exposure (Acute response chronic treatment) Levels of mRNA expression are reported relative to the expression in control flies. * $p<0.05$ compared to control, ANOVA with Bonferroni multiple comparisons. Bars represent SEM. n=4 independent experiments.

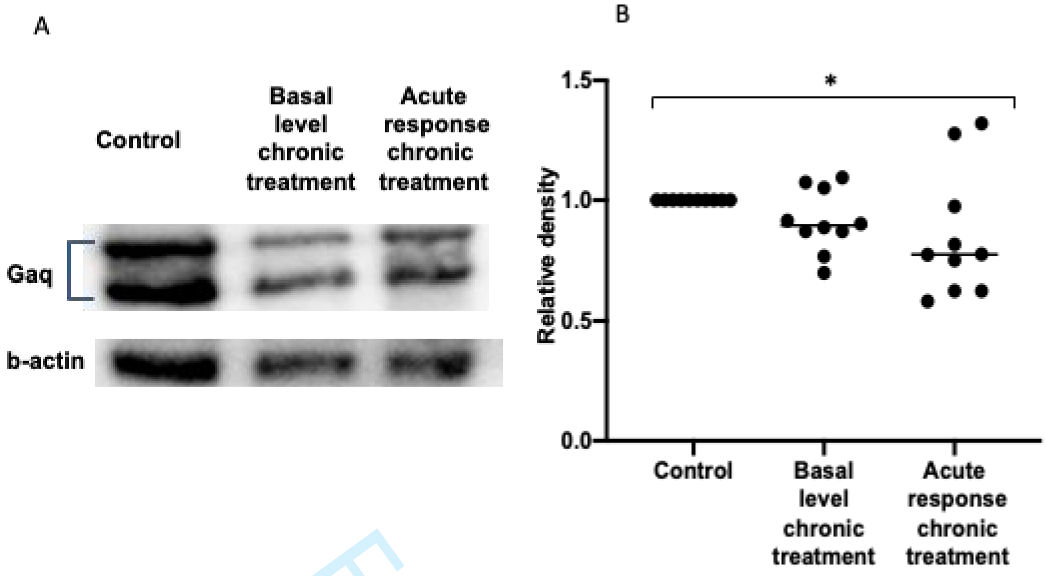


Figure 3 Western blot analysis of Gαq protein expression. Primary antibodies recognising Gαq and β-actin respectively were used to estimate relative levels of Gαq protein expression in *Drosophila* heads that had not been exposed to ethanol (control), sacrificed 24 h after two ethanol exposures (basal level chronic treatment) or 1 h after the third ethanol exposure (acute response chronic treatment). All flies were handled similarly and were sacrificed at the same time. Panel A: image of stained western blot membrane. Both bands recognised by the anti-Gαq were used for the calculation in panel B The lanes shown were selected from a larger gel (Full gels shown in Supplementary Figure 1). Panel B: quantification of Gαq protein levels (density of both bands added together) normalized to β-actin. Densities are expressed as a ratio to the control level in the same sets of bands as shown in panel A. Two separate experiments each consisting of duplicate sets of tubes of flies were treated as indicated (each tube containing 15 flies). The extracted samples were loaded in duplicates on gels and probed with polyclonal or monoclonal anti-Gαq antibodies. The band density of all ten sets of the three conditions (six stained with polyclonal and four with monoclonal antibodies) were measured and recorded as shown. Data was analysed by non-parametric Kruskal-Well test and showed overall a statistically significant decrease long horizontal bar * p = 0.0449. Short horizontal bars represent mean values.

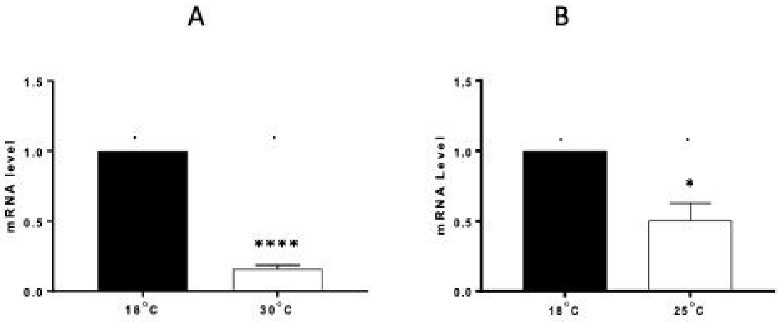


Figure 4 qRT-PCR of Gαq in knockdown mutant fly heads. Gαq mRNA was quantified by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method and normalized to an internal control β-actin. Measurements were carried out in (A) Tub-Gal4-Gal80ts-siRNAGαq (ubiquitous expression of Gαq siRNA) and (B) Elav-Gal4-Gal80ts-siRNAGαq (neuronal expression of Gαq siRNA) 72 h after the flies were transferred to a 30°C or 25°C incubator respectively. Results represent average of four independent experiments, with 6 flies per condition, and duplicate assays. t-test $*p < 0.05$, **** $p<0.0001$ Bars represent standard deviation.

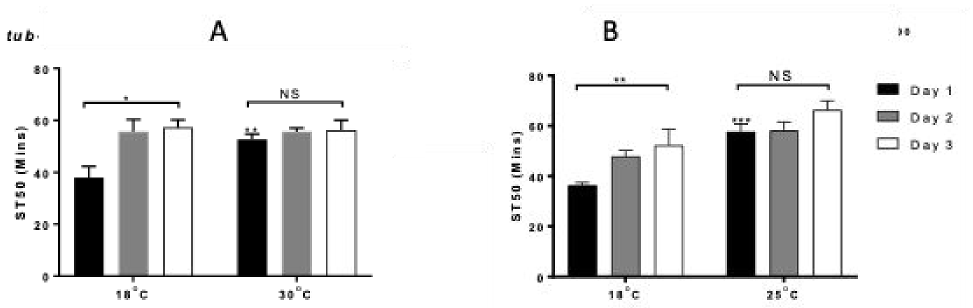
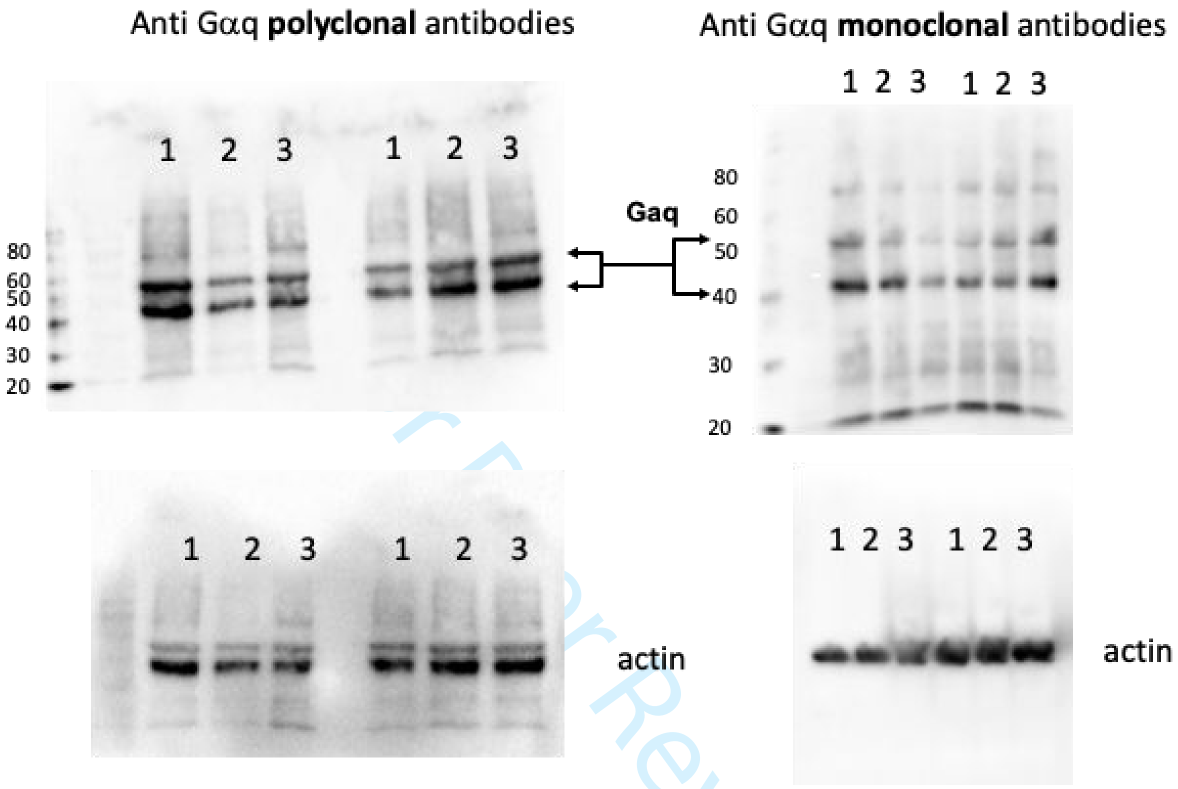


Figure 5 Sedation time (ST50) in *Drosophila* Gαq knockdown mutants. Groups of 8 male flies were incubated at either 18°C, 25°C or 30°C for 3 days, followed by exposure to 100% EtOH for 3 consecutive days, at the same time of the day. ST50 (time until 50% of the flies were sedated) was recorded. (A) w^{1118};tub−Gal80ts ; tub−Gal4/TM6c-Sb (ubiquitous expression of Gαq siRNA) and (B) w^{1118}/Y ;tub−Gal80ts ; elav−Gal4/TM6c-Sb (neuronal expression of Gαq siRNA). Horizontal bars indicate significance in ST50 change over three days. Stars over day 1 bars indicate significant difference between Day 1 30°C and Day 1 18°C or Day 1 25 °C and Day 1 18°C. ANOVA with Bonferroni post-hoc test *p<0.05; **p<0.01; ***p<0.001; NS=not significant. n=5-6 independent experiments. Bars represent standard deviation.

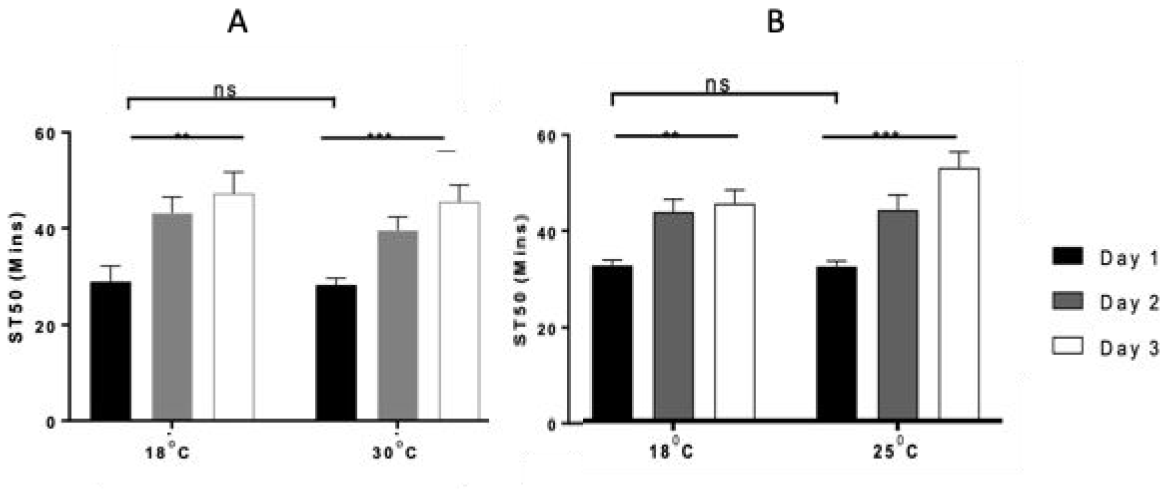
Supplementary Table 1

Gene	Day 1 vs Control	Day 3 vs Control	Primers
$G_{\gamma}1$	NAC	**	F: CGTTGCCGAGGAGTCAGCGA R: TCCAGGTGGCGTTGATACTGGT
$G_{\gamma}30A$	NAC	NAC	F: TCTGGTGCCGGTAGAGATGCAG R: TGAATGCTCCGCTTGCCCCC
Gβ5	NAC	NAC	F: TCTGGGACATGCGCTCTGGTCA R: TGCTGTCATCCGATCCAGTGGC
Gβ13F	NAC	NAC	F: CGTGGGTGATGACCTGTGCG R: CACGGGACACCCGGACGTTG
Gβ76C	NAC	NAC	F: ACCATCCCAGTGGCTTCGGGT R: GCCAGTGTTCTTCTGGGGCGG
Gαi	NAC	**	F: CGCGCAATGGGACGCCTGAA R: GCAGCAGGATGCCCTCGTCG
Gαq	NAC	***	F: CAGCAGCACGCGAAAGCGT R: GTCCCGGCGCAACTGCTTCT
Gαo	NAC	**	F: AACGCCTCTGGCAGGACG CC R: TTGGCGCCTAACCGATCCAAA
Gαf	NAC	NAC	F: CATGGGTGGTGGCGAACAGCAG R: CTGCACGAGATCAGGAACAATACGG
Gαs	NAC	*	F: AGCAGGATATTCTTCGGTGCCGT R: TTCCTACGCTCGTCCCGCTG

Supplementary Table 1. Effect of ethanol on G protein subunit expression. The expression of the indicated *Drosophila* G proteins was measured by qRT-PCR using the described specific primers in naïve *Drosophila* and *Drosophila* exposed to ethanol for 30 min for three consecutive days with 24 h interval. Experiments were repeated 2-3 times and qualitative comparisons adjusted to **β** actin expression are reported: NAC= no apparent change; * small changes (< 20%) in some repeats; ** changes (20-25%) in all repeats, larger changes (>25%) in all repeats. This data led to the choice of continuing work with Gαq, however some of the other subunits should be further investigated, especially G$_{\gamma}$1, Gαi and Gαo. (β-Actin primers were: F: GCGTCGGTCAATTCAATCTT and R:AAGCTGCAACCTCTTCGTCA)



Supplementary Figure 1. **Western blot analysis of Gαq protein expression.** Two full membranes are shown stained with either polyclonal or monoclonal anti-Gαq and reprobed with anti-actin antibodies. Lanes are numbered as : 1 = control, 2 = basal level chronic treatment, 3 = acute response chronic treatment as described in Figure 3.



Supplementary Figure 2. ST50 of background control flies. ST50 were measured in groups of 8 flies over 3 days. Panel A reports ST50 of flies resulting from crosses of w^{1118};tub−Gal80ts ; tub−Gal4/TM6c-Sb with Bloomington 36303 (control for Tub-Gal4-Gal80ts-siRNAGαq) and panel B reports ST50 of flies resulting from crosses of w^{1118}, elav-GAL4, mw+ ; tub-GAL80ts, mw+ ; + with Bloomington 36303 (control for Elav-Gal4-Gal80ts-siRNAGαq). There was no significant difference between day 1 at 18° vs 30°C or 18° vs 25°C while there was a significant increase in ST50 for all groups of flies. ANOVA with Bonferroni post-hoc test $^{**}p<0.01$; $^{***}p<0.001$; ns=not significant. n=3-4 independent experiments. Bars represent standard deviation.