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A histone modification identifies a DNA element controlling *slo* BK channel gene expression in muscle

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Abstract

The *slo* gene encodes BK type Ca^{2+} -activated K^{+} channels. In *Drosophila*, expression of *slo* is induced by organic solvent sedation (benzyl alcohol and ethanol) and this increase in neural *slo* expression contributes to the production of functional behavioral tolerance (inducible resistance) to these drugs. Within the *slo* promoter region, we observed that benzyl alcohol sedation produces a localized spike of histone acetylation over a 65 n conserved DNA element called 55b. Changes in histone acetylation are commonly the consequence of transcription factor activity and previously, a localized histone acetylation spike was used to successfully map a DNA element involved in benzyl alcohol-induced *slo* expression. To determine whether the 55b element was also involved in benzyl alcohol-induced neural expression of *slo* we deleted it from the endogenous *slo* gene by homologous recombination. Flies lacking the 55b element were normal with respect to basal and benzyl alcohol-induced neural *slo* expression, the capacity to acquire and maintain functional tolerance, their threshold for electrically-induced seizures and most *slo*-related behaviors. Removal of the 55b element did however increase the level of basal expression from the muscle/tracheal cell-specific *slo* core promoter and produced a slight increase in overall locomotor activity. We conclude that the 55b element is involved in control of *slo* expression from the muscle and tracheal-cell promoter but is not involved in the production of functional benzyl alcohol tolerance.

Introduction

The *slo* gene encodes the BK-type voltage- and Ca^{2+} -activated K^{+} channel that plays important roles in shaping action potentials, regulating neuronal firing patterns and synaptic transmission, and controlling smooth-muscle tone (Nelson, Krispel, Sekirnjak, & du Lac, 2003; Brayden & Nelson, 1992; Petkov et al., 2001; Hu et al., 2001). BK channels are evolutionarily conserved targets of organic-solvent action (Del Re, Dopico, & Woodward, 2006; Crowder, 2004; Dopico, Lemos, & Treisman, 1996). In *Drosophila*, the *slo* gene has

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also been implicated in the homeostatic neural adaptations that follow sedation by the organic solvents benzyl alcohol and ethanol (Ghezzi, Krishnan, & Atkinson, 2012; Cowmeadow et al., 2006; Cowmeadow, Krishnan, & Atkinson, 2005; Ghezzi, Pohl, Wang, & Atkinson, 2010; Ghezzi, Al-Hasan, Larios, Bohm, & Atkinson, 2004). In flies, activation of the gene appears to help counter the effects of these drugs by contributing to the development of functional tolerance (a reduced responsiveness to an effect of a drug caused by prior drug exposure). However, after drug clearance, the same increased *slo* activity that initially countered drug sedation persists to produce withdrawal symptoms, including enhanced seizure susceptibility (Ghezzi et al., 2010). Adult flies are ideal for the study of functional tolerance because they do not develop metabolic tolerance (increased rate of clearance) to these organic solvents (Ghezzi et al., 2010; Alhasan, 2009).

The *Drosophila slo* gene is regulated by alternative promoter activation and alternative mRNA splicing, both of which are thought to allow cells to fine tune the conductance and calcium sensitivity of the expressed channels. The 7 kb transcriptional control region of the *slo* gene is complex and contains at least five core promoters that mediate developmental- and tissue-specific expression (Bohm, Wang, Brenner, & Atkinson, 2000; Brenner & Atkinson, 1997; Chang et al., 2000; Yu, Upadhyaya, & Atkinson, 2006; Brenner, Thomas, Becker, & Atkinson, 1996). Within the *slo* transcriptional control region are two DNA elements, called 6b and 55b, that were originally postulated to be regulatory in nature because of their strong evolutionary-conservation within *Drosophila* species (Bohm et al., 2000; Chang et al., 2000). The 6b element is situated between the two neural promoters while the 55b element is located downstream of the neural promoters, near the muscle/tracheal cell-specific promoter of *slo*. A near-universal feature of transcription factors that activate gene expression is that they directly or indirectly produce a concomitant increase in local histone acetylation. Despite its proximity to the muscle/tracheal cell promoter, the 55b element was thought to be involved in benzyl alcohol induction of the neural promoters because of the appearance of a histone acetylation peak at 55b four hours after sedation (Wang, Krishnan, Ghezzi, Yin, & Atkinson, 2007). This peak at 55b appeared prior to induction of the gene. By 6 h post sedation, neural *slo* induction could be detected, and the region of enhanced acetylation no longer included 55b but overlaid the two neural promoters at the 5' end of the *slo* transcriptional control region. At 24 h post sedation, there was a single acetylation spike over the 6b element (Wang et al., 2007). After this time point, acetylation of the *slo* transcriptional control region and expression from the gene returned to baseline levels. The CREB transcription factor was linked to the production of both the 55b and 6b acetylation spikes. Mutant CREB transcription factors eliminated benzyl alcohol induction of these histone spikes, induction of *slo* expression, and the appearance of behavioral benzyl alcohol tolerance (Wang, Ghezzi, Yin, & Atkinson, 2009; Wang et al., 2007). From these observations we hypothesized that 55b and 6b were bound by transcription factors and that these transcription factors modulated the neural *slo* promoters in response to benzyl alcohol sedation in a way that contributed to benzyl alcohol tolerance. For the 6b element, this hypothesis was validated in that deletion of 6b specifically altered the response of the neural promoters to benzyl alcohol sedation and caused a behavioral change in response to benzyl alcohol exposure (Li, Ghezzi, Pohl, Bohm, & Atkinson, 2013). While basal expression was normal, it was seen that neural induction of *slo* by benzyl

alcohol sedation was substantially increased. Behaviorally, this resulted in a substantial increase in the duration of functional tolerance from about 10 days post sedation to greater than 28 days post sedation. In the current study, we genetically ablated the 55b element to determine the role it plays in baseline *slo* expression, in expression after benzyl alcohol sedation, and to determine the behavioral consequences of removal of this 55 n element.

Results

Generation of the 55b deletion mutant

To address the *in vivo* role of the 55b element, we created a knockout mutant by homologous recombination using ends-out gene targeting (Rong & Golic, 2000). A map of the transcriptional control region of the *slo* gene showing the position of the 55b element along with other evolutionarily conserved elements is shown in Figure 1A and the process used to delete the 55b element is schematized in Figure 1B. In brief, the 55b element was first substituted by a transgenic mini-*white*⁺ marker gene by homologous recombination to generate the *slo*^{w 55b} allele. Cre/lox recombination was then used to remove all transgenic material except for a single loxP (80n) cassette that replaced the 65 nucleotide 55b element to produce the *slo*^{55b} allele. In flies, loxP sites have not been observed to be transcriptionally active (Li et al., 2013). The fidelity of each step was confirmed by genomic Southern blotting (Figure 1C) and DNA sequencing (not shown). The derived *slo*^{55b} allele was then backcrossed to our wild-type (CS) stock for six generations to reduce differences in the genetic background. The *slo*^{55b} homozygotes are healthy animals that develop at normal rates and have normal longevity (data not shown). This is the same process that was previously used to ablate the 6b element (Li et al., 2013).

The 55b mutation specifically alters expression from the muscle/tracheal-cell promoter

The abundance of *slo* message in *slo*^{55b} flies was quantified by real-time RT-PCR using primers that specifically recognize products of the neuronal promoters. We found that the 55b deletion did not affect the basal expression level of *slo* neural isoforms (Figure 2) nor did it affect the induction magnitude or induction time course of the neural isoforms after benzyl alcohol sedation (Figure 3). For the neural isoforms, induction was detected 6 h after benzyl alcohol sedation and had disappeared by 24 h post sedation in both wild-type and 55b flies, whereas for the muscle isoform induction was not detected in either strain 6 h after benzyl alcohol sedation.

In *Drosophila*, muscle-specific BK channel expression is the product of the C2 core promoter [Figure 1A and Chang *et al.* (2000)]. The 55b element is about 300 n from the C2 transcription start site and might play a role in regulating C2 promoter activity in muscle. We also conducted quantitative RT-PCR using primers that specifically recognize the muscle-specific isoform produced from Promoter C2. We found that the *slo*^{55b} mutant exhibited significantly enhanced basal expression of the *slo* muscle-specific isoform compared to wild type (Figure 2B). Over the 24 h period of the experiment we did not observe a change in the basal expression from the C2 promoter from the mutant or wild type. However, in contrast to the transcriptional induction of *slo* neural isoforms, benzyl

alcohol exposure did not induce expression from the muscle-specific Promoter C2 in either wild-type or mutant lines (Figure 3E&F).

The 55b element is not required for benzyl alcohol-induced H4 acetylation changes

Histone acetylation is a common consequence of the action of transcription factors that stimulate gene expression. Because the earliest observed benzyl alcohol-induced histone H4 acetylation spike was centered over element 55b, we hypothesized that this acetylation increase might represent the first step in the benzyl alcohol-induced activation of the *slo* gene (Wang et al., 2007). If this hypothesis was correct, then loss of 55b might alter the induction process, changing the histone acetylation changes triggered by benzyl alcohol sedation.

To investigate whether factors associating with the 55b element contribute to benzyl alcohol-induced acetylation changes within the *slo* transcriptional control region, a chromatin-immunoprecipitation/quantitative-PCR assay was used to compare the benzyl alcohol histone H4 acetylation profiles from wild-type animals to the profiles from *slo*^{55b} mutant animals (Figure 4). Wild-type animals showed a profile in concert with that reported in Wang et al. (2007). At 4 h post sedation, a statistically significant increase in acetylation was observed over element 6b, neural promoter C1, and element 55b (see the map in Figure 1A). By 6 h after sedation, the peak at 55b was lost but the acetylation increase over neural promoter C0 had reached statistical significance. At 24 h after benzyl alcohol sedation, only the 6b element still showed a significantly increased histone acetylation state. The acetylation profile of the homozygous *slo*^{55b} mutants differed only subtly from the wild type acetylation profile. Specifically, in the *slo*^{55b} mutants, the histone peak over core promoter C0 achieved statistical significance slightly earlier (4 h post sedation) and the return to baseline acetylation was achieved slightly earlier (24 h post sedation). These small changes may be meaningless or may indicate a slight advancement in the time course of the response to benzyl alcohol. However, based on these data, it seems that the 55b element is not required for subsequent benzyl alcohol-induced histone acetylation across the *slo* promoter region; that is, the 55b element is not a trigger for the later histone modifications.

slo^{55b} does not affect benzyl alcohol-related behavioral responses

As assayed in fly heads, the *slo*^{55b} mutation did not affect the benzyl alcohol-induced *slo* induction and only subtly altered the benzyl alcohol-induced histone acetylation profile of *slo*. Nonetheless, it is still possible that the *slo*^{55b} mutation could alter behavioral responses to alcohols if it impeded function in a region of the brain that underlies the behavior. Therefore, *slo*^{55b} homozygous animals were subjected to a battery of drug-related behavioral tests, all of which have been shown to be sensitive to changes in *slo* expression. The behaviors examined were benzyl alcohol resistance, tolerance, and withdrawal-induced hyperexcitability. Resistance refers to the innate basal drug sensitivity while tolerance refers to the drug-inducible increase in drug resistance. Tolerance is believed to be a homeostatic response to an effect of the drug that counters the effects of the drug. Previously, benzyl alcohol-induced *slo* expression has been shown to underlie tolerance to this drug (Ghezzi et al., 2004) and transgenic induction of *slo* has been shown to phenocopy this response of generating benzyl alcohol resistance. Withdrawal-induced neuronal hyperexcitability that is

normally apparent 24 h after benzyl alcohol sedation has also been shown to be dependent on benzyl alcohol-induced changes in *slo* gene expression (Ghezzi et al., 2010).

We used our standard recovery-from-sedation assay to determine if the *slo*^{55b} mutation altered the resistance or tolerance phenotypes of flies to benzyl alcohol. Age-matched female wild-type flies (Canton S line) and *slo*^{55b} animals were exposed to a sedating dose of benzyl alcohol. Once sedated, they were allowed to recover in a fresh-air environment and the rate of recovery of the population was monitored. The recovery rate of *slo*^{55b} homozygotes was indistinguishable from that of the wild-type animals. This indicates that the *slo*^{55b} mutation does not alter benzyl alcohol resistance (Figure 5).

The capacity to acquire benzyl alcohol tolerance of the mutant and wild type were also compared. Because deletion of the 6b element from the *slo* promoter region had been shown to affect the duration of functional tolerance, we performed a time-course analysis of tolerance with the *slo*^{55b} mutants (Li et al., 2013). In this assay, one group of animals is mock sedated while a matched group is sedated with benzyl alcohol vapor. After sedation, the animals are allowed to recover in a fresh-air environment and stored on food. One, seven, or fourteen days later, both groups were sedated with benzyl alcohol vapor, switched to a fresh-air environment, and monitored for their rate of recovery. Stocks were said to be capable of acquiring tolerance if they recovered more rapidly from their second sedation than from their first sedation. As shown in Figure 6, both the wild type and *slo*^{55b} homozygotes displayed tolerance that was apparent one day after the first sedation event. For both stocks, tolerance persisted for seven days but was lost 14 days post sedation. This is in agreement with previous measures of the duration of benzyl alcohol tolerance by wild-type flies. These results suggest that, unlike the 6b element, the 55b element does not affect the acquisition or the duration of tolerance to sedation with benzyl alcohol.

Benzyl alcohol-induced *slo* expression acts as a neural excitant that counters the sedative effects of the anesthetic (Ghezzi et al., 2010). However, after benzyl alcohol clearance, the *slo*-induced increase in excitability persists to generate a withdrawal-related reduction in seizure threshold. This seizure phenotype is caused by a reduction in the neuronal refractory period that increases the capacity for repetitive neuronal firing (Ghezzi et al., 2010). To investigate whether the *slo*^{55b} mutation alters benzyl alcohol-induced effects on the refractory period of the giant fiber pathway, we measured the giant fiber following frequency in homozygous mutant animals. Trains of stimuli at various frequencies ranging from 40 to 220 Hz were delivered to the brain, and responses in the flight muscle were recorded. The giant fiber circuit fails to respond when the interval between the individual stimuli is less than the refractory period of the giant fiber neurons. We found that the FF50 (the frequency with 50% response rate) of giant fiber pathway of the mutant and wild type had the same baseline levels, and both were elevated to the same degree 24 h after benzyl alcohol sedation (Figure 7A).

To further characterize the neural excitability of the flies, trains of high-frequency electroconvulsive shocks (ECS) of various voltages were delivered to the brain to trigger a seizure-like electrical response, and the minimum triggering voltage was recorded as the seizure threshold. The seizure-like phenotype was characterized as a high-frequency

spontaneous initial discharge followed by a failure period to the response and a delayed secondary discharge (Figure 7B). The average seizure stimulus voltages in the mutant and wild-type flies were measured before and after benzyl alcohol sedation. Without benzyl alcohol exposure, the *slo*^{55b} flies exhibited an average threshold of 33.18 ± 3.182 V, while the stimulating voltage dropped to 21.88 ± 2.98 V one day after benzyl alcohol sedation (Figure 7C). The wild type showed similar responses, suggesting that the mutation does not affect basal seizure susceptibility nor the benzyl alcohol-induced enhancement of seizure susceptibility.

The 55b mutation does not alters circadian rhythmicity

Flies homozygous for null mutations in the *slo* gene lack normal circadian rhythms. This phenotype is thought to arise because *slo*-encoded BK channels are functionally important in a neuronal output from the central circadian pacemaker (Fernandez et al., 2007). We compared the circadian rhythmicity of *slo*^{55b} homozygotes to wild type animals and to animals homozygous for the *slo*⁴ null allele under free-running conditions (Figure 8). As shown in Figure 8A&B, the *slo*^{55b} homozygotes showed normal circadian locomotor rhythmicity (rhythm index wild type: 0.33 ± 0.02 ; *slo*^{55b} 0.35 ± 0.02), while *slo*⁴ null mutants were arrhythmic (rhythm index 0.08 ± 0.03). The period length of wild type, *slo*^{55b}, and *slo*⁴ animals did not differ, although *slo*⁴ animals individually showed much greater variation in period length (Figure 8C). Panel 8D shows that sleep, which is related to circadian rhythmicity, also appears unaffected by the *slo*^{55b} mutation (within the limits of its definition in flies—absence of movement for a 5 minute epoch).

The 55b mutation specifically alters basal activity but does not produce incoordination

Basal mobility is an important indicator of normal physiological performance, and since the deletion of 55b specifically alters muscle *slo* expression, we also explored more generic locomotor-related phenotypes. We observed that the *slo*^{55b} homozygous animals are indistinguishable from wild type in climbing assays and in a simple flight assay (Figure 9A and B). Furthermore, *slo*^{55b} homozygotes did not show the sticky-feet phenotype (data not shown) that is caused by a loss of *slo* expression in the nervous system (Atkinson et al., 2000). However, in the free-running activity plot (Figure 8A), one apparent difference between *slo*^{55b} mutant and the wild type is that the *slo*^{55b} mutants show increased magnitude in both the evening and morning peaks of activity. This is more clearly seen in Figure 9C–E. Deletion of the 55b element resulted in a pronounced increase in morning and evening peaks of activity (Figure 9C–E) when compared to age-matched wild type control animals, albeit this increase was not evident when net daily activity was measured (Figure 9F).

Discussion

In Wang et al. (2007) we showed that, within the *slo* promoter region, benzyl alcohol sedation produced histone H4 acetylation spikes at the evolutionary-conserved DNA elements 55b and 6b. Furthermore, acetylation at these locations and *slo* induction were sensitive to mutations affecting the CREB2 transcription factor. These observations led us to the proposal that both DNA elements were functionally important in benzyl alcohol

induction of the *slo* gene. For the 6b element this prediction was borne out in that removal of the 6b caused the *slo* gene to respond more profoundly to benzyl alcohol sedation which produced longer-lasting benzyl alcohol tolerance (Li et al., 2013). Because the 55b histone acetylation peak preceded the acetylation peak at 6b we predicted that the 55b element participated in a very early step in *slo* induction. We expected its removal to eliminate the response of *slo* to benzyl alcohol sedation. Nevertheless, deletion of the 55b element from the endogenous gene did not appear to interfere with net benzyl alcohol-induced changes in histone H4 acetylation (within the *slo* promoter region), gross benzyl alcohol-induction of the gene, the appearance of *slo*-mediated functional behavioral benzyl alcohol tolerance, or the *slo*-mediated benzyl alcohol withdrawal responses. This clearly indicates that, unlike the 6b element, the 55b element is not important in the benzyl alcohol-induced tolerance. Our simple approach of using drug-induced acetylation changes to identify candidate regulatory elements worked for one of two candidate elements (6b but not 55b). At this time we do not know the function of the benzyl alcohol-induced histone H4 acetylation peak over the 55b element. It may be that benzyl alcohol causes a transcription factor/histone acetylase complex to acetylate 55b in most cells. Increased histone acetylation makes the underlying DNA more accessible (Lee, Hayes, Pruss, & Wolffe, 1993) and both activating and repressing transcription factors are dependent on access to the underlying DNA in order to function. While it is usual to think that increased and decreased histone acetylation is directly correlated with increased and decreased expression, it is possible that in some neurons, the more accessible underlying DNA is bound by an activator while in other cells it is bound by a repressor. Thus, we might not detect a change in activity from the neural promoters solely because the RNA is prepared from a homogenate. Despite this limitation, we can still conclude that the function of the 55b element is not essential for the benzyl-alcohol induced change in *slo* expression that underlies benzyl alcohol behavioral tolerance. Although we performed a large variety of behavioral tests it is possible that a 55b-mediated change has additional behavioral consequences that we have not considered and that do not show up in any of our tests.

Removal of the 55b element appears to cause a slight increase in the basal activity of the muscle-specific core promoter [a phenotype not produced by the removal of the 6b element (Li et al., 2013)]. This core promoter is called promoter C2, and it has been extensively characterized as being responsible for muscle expression throughout development and for tracheal cell expression in (at least) the embryo and larval stages (Brenner et al., 1996; Chang et al., 2000; Thomas, Wang, Brenner, & Atkinson, 1997). Promoter C2 activity in the wild type is not responsive to benzyl alcohol sedation—a property that remains unchanged by the 55b deletion mutation. Promoter C2 has not been observed to be active in the nervous system. It is therefore not surprising that a change in its activity does not affect the capacity to acquire functional benzyl alcohol tolerance—a phenotype that is dependent on expression of *slo* in the nervous system (Ghezzi et al., 2004). The only behavioral effect ascribed to the 55b deletion mutation was an increase in baseline motility. This increase corresponded to the normal times of increased activity (morning and evening) which is expected since the mutation does not affect circadian rhythmicity.

The evidence presented here suggests that the 55b element has a slight influence on expression from Promoter C2. However, our measurement of expression level is based on whole-animal RNA and therefore reflects an average level of expression. Measurements of this kind cannot detect changes that involve a small number of cells and would also be blind to reductions or increases in expression in one part of the animal that are matched by the opposite change in other parts of the animal.

Despite the small apparent change in expression produced by the 55b deletion, it is very likely that 55b is a regulatory element involved in regulating some aspect of *slo* expression. Wang et al. (2007; 2009) showed that 55b is one of three sites in the *slo* transcriptional control region that is bound by the CREB transcription factor. While 55b does not contain a canonical CREB binding site (CRE) it does contain a putative AP-1 binding site. The CRE sites and AP-1 sites share consensus DNA sequences, and in mammals CREB has been shown to bind AP-1 binding sites (Newell, Deisseroth, & Lopez-Berestein, 1994; Hai & Curran, 1991; Masquilier & Sassone-Corsi, 1992). In addition, Modencode data show that the 55b element underlies a CREB binding protein (CBP) peak (CBP is a histone acetyl transferase that is recruited by CREB and other transcription factors). During embryogenesis, Modencode also shows that the 55b element underlies a peak of binding by the *Distal-less* (*Dll*) transcription factor (Roy et al., 2010). Finally, 55b also contains two DNA motifs that match the site recognized by the *dorsal* (*dl*) transcription factor, albeit no physical evidence of *dl*-binding exists (Wang et al., 2007). Despite these interactions, the 55b element does not appear to be important for the homeostatic response to sedation with the organic solvent anesthetic benzyl alcohol and it remains unclear why it is the site of a benzyl alcohol-induced histone acetylation spike.

Methods

Fly stocks

Drosophila stocks were Canton S (wild type), *slo*⁴ and *slo*^{55b}. Flies were raised on standard cornmeal medium in a 12:12 light:dark cycle at 22 °C. For behavioral assays, newly eclosed flies were collected and tested 3–5 days after eclosion.

Ends-out gene targeting of the 55b element

The 55b element of *slo* was ablated using ends-out gene targeting method developed by Golic and coworkers (Gong & Golic, 2003). Two homologous regions flanking the *slo* 55b element were PCR amplified from Canton S genomic DNA using the proofreading *PfuTurbo* DNA polymerase (Stratagene CA). The primers used were 5'-GCGGCCGCCTCGGTGGTTAGCCAGTA-3' and 5'-GCGGCCGCGCCAAGACAAGGCGAATTCAA-3' which added *NotI* sites to both ends of a 3.1-kb DNA fragment upstream of the 55b element, while the primers 5'-GGCGCGCCAAATGCCCGTATAGTCATA-3' and 5'-GGCGCGCCTAAAGACGCCAGACAAATG-3' were used to amplify and add *AscI* termini to a 3-kb DNA fragment downstream of 55b. These two fragments were inserted into the ends-out vector pW25 in the same orientation flanking the mini-*white*⁺ gene carried by the vector (Gong & Golic, 2004). This donor construct was introduced into the *white*⁻ fly

by *P* element germline transformation. Lines with the donor transgene on the first or the second chromosome were used to induce gene targeting.

To induce targeting, donor insertion lines were crossed to the *P{hsp70FLP}P{hsp70I-SceI}* strain (Bloomington # 6934), and the progeny were heat-shocked at 38°C for one hour during their first three days of development to stimulate transgenic FLP and I-SceI expression which act together to stimulate homologous gene replacement targeting at the 55b region. Virgin females carrying *w*⁺ and *P{hsp70FLP}P{hsp70I-SceI}* were crossed to male FLP flies (Bloomington # 6938) and those that produced solid red-eyed progeny eliminated (mini-white gene had not been mobilized). In the remainders, the *white*⁺ gene was genetically mapped to identify targeted events in which *white*⁺ had relocated to the target chromosome 3. Finally, the targeted strain was crossed to *P{Cre}* line (Bloomington # 1501) to remove the *white*⁺ maker and obtain the 55b deletion mutant *slo*^{55b}. Female *slo*^{55b} flies were backcrossed to Canton S males for six generations to reduce differences between the mutant and wild-type control stocks. The sequence of the modified gene has the Genbank accession # KM283199.

Southern blotting analysis

Southern blot analyses were performed to verify the ends-out targeting events using a DIG kit (Roche Diagnostics, IN) as recommended by the manufacturer. Genomic DNA was prepared from flies carrying the targeted alleles and wild type, followed by overnight digestion with *HindIII* (NEB, MA), separated by agarose gel electrophoresis and transferred to nylon membranes as described (Ausubel, 2001). The probe was the 3.1-kb PCR fragment immediately upstream of the 55b element. The blot was hybridized to the digoxigenin (DIG) labeled probe and was detected by DIG antibody and chemiluminescence signal. DNA sequencing was used to confirm the identity of the final gene replacement product.

Benzyl alcohol tolerance assay

Thirty ml glass vials were coated with 200 µl of 0.4% benzyl alcohol in acetone, followed by constant rotation for 20 min to evaporate the highly volatile acetone and leave a finely distributed thin layer of the less volatile benzyl alcohol on the wall. The vials for controls (mock sedation) were coated with acetone only, which completely evaporated after 20 min. Two groups of age-matched (3–5 day old) female flies were prepared. One group was transferred into the benzyl alcohol-coated vial while the other group was transferred into the control vials where they remained until all the flies in the drug vials were sedated (usually 5–10 min). All vials contained 10 females. After exposure, both groups were moved back to food vials to recover for 1 day, 7 days, or 14 days. At this time, both groups were sedated in benzyl alcohol-coated vials. Following sedation, their rate of recovery in a fresh air environment was measured as described. Flies were scored as recovered when they resumed climbing. The recovery was analyzed by a computer-based movement detection system (Ramazani, Krishnan, Bergeson, & Atkinson, 2007). The recovery curves were plotted as the percentage of recovered flies against time.

Benzyl alcohol resistance assay

Benzyl alcohol-coated glass vials were prepared as described above. Three to five day old female wild type (Canton S) flies and *slo*^{55b} were sedated in parallel (10 flies per vial). After sedation, the recovery rate in a fresh-air environment was measured as described above.

Quantitative RT-PCR analysis

RNA was extracted from whole animals (Ghezzi et al., 2004). Quantitative RT-PCR was conducted as described (Cowmeadow et al., 2006). To measure expression from the neural specific core promoters, the primers used were: *slo* exon C1 forward primer 5'-AAACAAAGCTAAATAAGTTGTGAAAGGA-3'; *slo* exon C1 reverse primer 5'-GATAGTTGTTTCGTTCTTTTGAATTTGA-3'. To measure expression from the muscle/tracheal cell-specific core promoter the primers used were: *slo* exon C2 forward primer 5'-GCTATTTATAATAGACGGGCCAAGTT-3'; *slo* exon C2 reverse primer 5'-GGAAATCCGAAAGATACGAATGAT-3'. Internal control primers were: *Cyp1* forward primer 5'-ACCAACCACAACGGCACTG-3 and *Cyp1* reverse primer 5'-TGCTTCAGCTCGAAGTTCTCATC-3'. Normalization was as described in (Ghezzi et al., 2004; Cowmeadow et al., 2006). To determine the effect of benzyl alcohol sedation on *slo* expression we compared benzyl alcohol sedated and mock sedated (control) flies as described in the benzyl alcohol tolerance assay section (above).

Chromatin immunoprecipitation assay

About 1,500 flies were either benzyl alcohol sedated or mock sedated for 6–8 minutes and were allowed to recover in a drug free environment. Six and twenty-four hours after sedation, flies were frozen in liquid nitrogen, and fly heads were harvested by vortex decapitation and sieving. Heads were cross-linked with 2% formaldehyde for 3 min and chromatin was solubilized and sonicated on ice by 5 sections of 25 s sonication bursts to produce fragments of ~600 bp using a sonic Dismembrator 250 (Fisher Scientific). The chromatin immunoprecipitation assays were performed as described (Wang et al., 2007). The polyclonal antibodies against acetylated histone H4 at K5, K8, K12 and K16 (Upstate Biotechnology catalog # 06-866) was used to precipitate DNA. Chromatin immunoprecipitation assays were performed more than three times with independent chromatin samples.

Climbing assay

The climbing assay was conducted to measure relative activity of flies as described (Feany & Bender, 2000). A group of 12 flies were placed in a 20 × 150 mm glass test tube, and were gently tapped to the bottom. The number of flies that climbed beyond 10 cm within 10 s was counted. The experiment was repeated three times. Statistical significance was calculated using the Student's *t* test.

Flight assay

The flight capacity of animals was determined with minor modifications as described (Brenner et al., 2000). Approximately 500 flies were dumped into a 15 × 62 cm pipette jar

through a funnel at the top. The falling flies flew toward the wall and were stuck in the mineral oil pre-coated on the wall. The position of each fly was marked and its distance from the top was measured. Flies with good flight capacity clustered near the top of the jar, whereas flies that flew poorly stayed close to the bottom.

Sticky-feet behavioral assay

The sticky-feet behavioral test was performed as described (Atkinson et al., 2000). Four-to-five-day old flies were heat shocked in a glass vial at 37°C for 7 min. Animals were gently transferred to the benchtop and left undisturbed for about 10 s. A flat toothpick was used to gently push flies on their side. The sticky-feet phenotype is said to occur when a fly holds onto the surface instead of flying or moving away.

Circadian rhythm analysis and general activity measurement

Flies were raised in a rhythmic 12:12 light:dark cycle. Male flies (aged 4–6d) were individually loaded, without anesthesia, into 5 mm × 65 mm glass tubes with 5% sucrose 2% agar food at one end. These tubes were then loaded into DAM2 *Drosophila* Activity Monitors (Trikinetics, Waltham MA) that have infrared beams that detect when a fly crosses the middle of the tube. The monitors are then placed into an incubator kept at 24°C. At the end of the light cycle after the flies are placed into the incubator, the lights are permanently turned off to observe free-running rhythms. Flies were monitored in 5-minute bins for 6 days. Any fly that did not move for any 24-hour period was discarded from analysis. Circadian analysis was analyzed using a Matlab software (Levine, Funes, Dowse, & Hall, 2002). Only the first week of activity was analyzed. Plots showing movement over time are created by averaging movement from flies of each genotype. Period and rhythm indices were generated by autocorrelation as described.

Activity throughout the day was determined by counting DAM2 beam crosses (30 minute bins) per day. Morning and evening peak activity (number of beam crosses per 30 minute bin) were determined by extracting the maxima from the 6 am to 10 am window and the maxima from the 6 pm to 10 pm window, respectively. Net daily activity was the total number of DAM2 beam crossings per day.

Electrophysiological analysis

The following frequency and the seizure threshold of the giant fiber pathway was measured utilizing the giant fiber pathway as described in detail in Ghezzi et al. (2010). Briefly, electrolytically sharpened tungsten electrodes (FHC, Inc) were placed on the compound eyes, and a recording electrode was placed through the dorsal cuticle into the right-upper DLM. The following frequency was measured by gradually increasing the stimulus frequency (with interspersed rest periods) until the giant fiber pathway failed to follow. Seizure threshold was measured with a 1.5-second 200 Hz electroconvulsive shock of increasing voltage. A seizure was recognized as a high frequency initial discharge, followed by a prolonged failure period and a delayed secondary pulse produced by the nervous system. The minimum voltage of stimulation to trigger seizure was determined as seizure-voltage threshold of the fly. Significance was determined using the Student's *t* test.

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Figure 1.

Generation of the 55b deletion mutant. A) Map of the ~7kb *slo* transcriptional control region. Bent arrows on the line are transcription start sites for the five identified tissue specific core promoters that are named C0, C1, C1b, C1c, and C2. The gray boxes on the line are the first exon expressed from each core promoter. The short black box on the far right represents the first exon common to all *slo* transcripts. Red boxes below the line are short sequences conserved among *Drosophila* species. B) Homologous recombination strategy used to generate the transgenic lines *slo*^{w 55b} and *slo*^{Δ55b}. Homologous recombination between the replacement DNA (pink) and the chromosome (white) substitutes the 55b element with a floxed mini-*white* gene that is called *slo*^{w 55b}. Cre recombinase was used to excise the mini-*white* gene to produce the *slo*^{Δ55b} mutant allele. C) Genomic Southern blot analysis was used to confirm the procession described in B). Genomic DNA was digested with *Hind*III and Southern blot analysis was performed using a DIG-labeled probe that specifically recognizes the 3 kb upstream DNA sequence of 55b. Lane 1, DNA standard. Lane 2, DNA from wild-type flies displayed a ~5.2 kb *Hind*III band. Lane 3, DNA from a *slo*^{w 55b} homozygote that carries the mini-*white* insertion produces a

band of ~10 kb. Lane 4, DNA from the *slo*^{55b} produced by excision of the mini-*white* gene from the *slo*^{w 55b} allele generates a band of ~5.3 kb.

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Figure 2.

The *slo*^{55b} mutation affects basal expression from the muscle/tracheal cell promoter but does not affect basal expression from the neural promoters of *slo*. Relative *slo* expression levels in wild-type and *slo*^{55b} stocks determined by real-time RT-PCR using either C1 primers that detect transcripts from both neural promoters (Panel A) or C2 primers that detect transcripts from the muscle/tracheal cell promoter (Panel B). A) Basal expression of neural promoter specific transcripts is not altered by the *slo*^{55b} mutation. B) Basal expression from muscle/tracheal-specific promoter is altered by the *slo*^{55b} mutation. Transcript abundance is expressed relative to the wild type. Statistical significance was determined by Student's *t* test (*n* = 3, **P* < 0.05). Error bars represent SEM.

Figure 3.

Benzyl alcohol induction of *slo* does not differ between wild-type and *slo*^{55b} stocks. Relative *slo* expression levels in wild-type and *slo*^{55b} stocks determined by real-time RT-PCR using either C1 primers that detect transcripts from the neural promoters (Panels A–D) or C2 primers that detect transcripts from the muscle/tracheal cell promoter (Panel E–F). A–D) Benzyl alcohol induction of *slo* neuronal–promoter expression of wild-type and *slo*^{55b} stocks is similar. E, F) Benzyl alcohol does not induce expression of the muscle/tracheal cell promoter in wild-type or *slo*^{55b} stocks. Transcript abundance is expressed relative to mock-sedated control animals. Statistical significance was determined by Student's *t* test (*n* = 3, **P* < 0.05). Error bars represent SEM.

Figure 4.

Patterns of histone H4 acetylation across *slo* transcriptional control region in *slo*^{55b} homozygotes after benzyl alcohol (BA) sedation. A) Map of *slo* control region as described in Figure 1A. In panels B-D the fold change of acetylation was determined by chromatin immunoprecipitation followed by real-time PCR using primers specific to the distinct conserved elements depicted as red boxes in (A). Change in acetylation is expressed as the ratio of the acetylation levels in benzyl alcohol-sedated flies and untreated flies. B) H4 acetylation levels at 4 h after benzyl alcohol sedation. C) Acetylation levels at 6 h after benzyl alcohol sedation. D) Acetylation levels at 24 h after benzyl alcohol sedation. Statistical significance was determined by one-way ANOVA with Dunnett's comparison post tests ($n = 3$, $*P = 0.05$) but error bars are SEM.

Figure 5.

The 55b deletion mutation did not affect resistance to benzyl alcohol sedation. To test for differences in the resistance to sedation with benzyl alcohol, the rate of recovery from a single benzyl alcohol sedation of wild type and *slo*^{55b} mutant flies was compared. Age-matched female flies were sedated with benzyl alcohol vapor and transferred to a fresh air environment, and the percentage of recovered flies over time was recorded. Both the wild-type and *slo*^{55b} flies recovered at the same rate. The statistical significance between the two recovery curves was determined by log-rank test ($n = 5$, $P = 0.1949$) and error bars represent SEM.

Figure 6.

The 55b deletion mutant acquires normal tolerance to benzyl alcohol induced sedation. Each curve shows the recovery time course of flies after a single benzyl alcohol sedation (blue) and following their second sedation (red). Canton S wild type (WT) was utilized as a control to illustrate tolerance with normal duration. In both the wild-type and *s/o*^{55b} lines, tolerance was detectable 1 day after the first exposure, 7 days after the first exposure, but had disappeared by 14 days after the first exposure. The statistical significance between the two recovery curves was determined by log-rank test ($n = 5-6$, P values are presented on the each graph).

Figure 7.

Benzyl alcohol sedation caused similar electrophysiological changes in *slo*^{55b} and wild-type flies. A) Benzyl alcohol (BA) sedation increases the giant fiber following frequency of wild type (WT) and mutant (*slo*^{55b}) animals to a similar degree. B) A stereotypical seizure response is shown. A constant low-frequency stimulus was applied throughout the duration of the recording to assess the responsiveness of the giant fiber pathway. An electroconvulsive stimulus (E) triggers a high-frequency initial discharge (ID), followed by a period of evoked response failures (Failure), and a delayed discharge (DD). After the delayed discharge the giant fiber has recovered (R) and responds normally to the low frequency stimulus. C) Benzyl alcohol (BA) sedation enhanced seizure susceptibility in both lines. Electroconvulsive stimuli of varying voltages ranging 5–50 V were utilized to

determine seizure susceptibility, which is represented by the minimum stimulus voltage to trigger seizure. The average stimulus voltages in wild type and *slo*^{55b} measured one day after benzyl alcohol (BA) sedation. The difference in seizure susceptibility in wild type and mutant animals does not differ. Unpaired Student *t* test, *n* = 5~9, **P* < 0.05. Error bars represent SEM.

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Figure 8. The *slo*^{55b} mutation did not affect circadian rhythmicity

A) Actograms of wild-type, *slo*^{55b}, and *slo*⁴ flies. Recently eclosed flies were entrained in 12:12 light:dark for 3 days before monitoring their free-running activity (beam crossings per 5 minute bins) under constant darkness conditions for six consecutive days. Both *CS* and *slo*^{55b} flies demonstrated rhythmic oscillation in activity, while *slo*⁴ was behaviorally arrhythmic. Rhythm index, period and sleep were quantified for each fly throughout the duration of the activity measurement. One-way ANOVA with Dunnett's post hoc test was used to determine statistical significance. Error bars represent SEM. **P* < 0.05; ***P* < 0.01.

Figure 9. The *slo*^{55b} mutation specifically alters basal locomotor activity

A) Climbing test: Homozygous *slo*^{55b} flies showed normal climbing ability. Over 95% of Canton S wild type (WT) and *slo*^{55b} flies passed the climbing test, whereas only 30% of the loss-of-function mutant *slo*⁴ stock passed it. B) Flight test: The *slo*^{55b} mutation did not affect flight capacity. Flies were dropped into a 15 × 62 cm pipette jar at the top. The falling flies flew toward the wall and were stuck in the mineral oil pre-coated on the wall, and the distance from the top was measured. Wild-type and *slo*^{55b} animals fly well, and most of them are found within 10 cm of the top. In contrast, *slo*⁴ flies flew poorly and were evenly distributed along the wall. C–F) Daily activity profile: The *slo*⁴ null allele cause animals to be active all of the time whereas the *slo*^{55b} mutation only increases the magnitude of the morning and evening activity peaks but not the time of their appearance. This difference between the wild type and *slo*^{55b} is not apparent in the net daily activity measurement. C) Daily activity plotted at distinct times of the day (organized in 30 minute bins), averaged over the first 4 consecutive days in free running conditions (dark-dark). D) Average magnitude of morning peak activity. E) Average magnitude of evening peak activity. F) Average net daily activity. Error bars represent SEM. One-way ANOVA with Dunnett's post hoc test was used to determine statistical significance: *P 0.05; **P 0.01; ***P 0.001.