A Genetic Analysis of the *stoned* Locus and Its Interaction With *dunce*, *shibire* and *Suppressor of stoned* Variants of *Drosophila melanogaster*

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ABSTRACT

The genetic complementation patterns of both behavioral and lethal alleles at the *stoned* locus have been characterized. Mosaic analysis of a *stoned* lethal allele suggests that *stoned* functions either in the nervous system or in both the nervous sytem and musculature, but is not required for gross neural development. The behavioral alleles stn^{ts} and stn^{C}, appear to be defective in a diametrically opposite sense, show interallelic complementation, and indicate distinct roles for the *stoned* gene product in the visual system and in motor coordination. A number of other neurological mutations have been investigated for their possible interaction with the viable *stoned* alleles. Mutations at two loci, *dunce* and *shibire*, act synergistically with the stn^{ts} mutations to cause lethality, but fail to interact with stn^{C}. A third variant (*Suppressor of stoned*) has been identified which can suppress the debilitation associated with the stn^{ts} mutations. These data, together with a previously identified interaction between the stn^{ts} and *tan* mutants, indicate a central role for the *stoned* gene product in neuronal function, and suggests that the *stoned* gene product interacts, either directly or indirectly, with the neural cAMP second messenger system, with the synaptic membrane recycling pathway via dynamin, and with biogenic amine metabolism.

THE use of behavioral mutants in *Drosophila melanogaster* to dissect various aspects of nervous system function has had a major role in the elucidation of the molecular mechanisms underlying membrane excitability and synaptic transmission. The identification of the *Shaker* locus as a gene encoding a form of voltage-activated K^+ channel (JAN, JAN and DENNIS 1977; SALKOFF 1983; PAPAZIAN *et al.* 1987; PONGS *et al.* 1988), had a major impact on the way we perceive rectifying currents in both the invertebrate and vertebrate nervous system. Similarly the characterization of the learning mutants *dunce* and *rutabaga* as being defective in forms of cyclic AMP phosphodiesterase (BYERS, DAVIS and KIGER 1981) and adenylate cyclase (LIVINGSTONE, SZIBER and QUINN 1984; LIVINGSTONE 1985) respectively, implicated the cAMP second messenger system in the learning process.

Another group of potential neurological mutants was isolated in a screen for temperature-sensitive paralytic mutants (GRIGLIATTI *et al.* 1973). This screen identified three *X*-linked loci that could mutate to produce high temperature-induced, but reversible, adult paralysis. Of these loci the *paralytic* ($para^{ts}$) and *shibire* (shi^{ts}) genes have been cloned and sequenced. The *paralytic* gene has been shown to encode one form of voltage-sensitive Na^+ channel (LOUGHNEY, KREBER and GANETSKY 1989). The *shibire* gene encodes the Drosophila equivalent of mammalian dynamin (VAN DER BLIEK and MEYEROWITZ 1991; CHEN *et al.* 1991), which implicates this microtubule associated protein with membrane recycling and endocytosis (KOSADA and IKEDA 1983; KOENIG and IKEDA 1989; KESSEL, HOLST and ROTH 1989; TSURUHARA, KOENIG and IKEDA 1990; MASUR, KIM and WU 1990). The third gene identified in this screen was *stoned*. The *stoned* locus was mapped by recombination analysis at 66.3 (GRIGLIATTI *et al.* 1973), and more recently, fine structure mapped using deletions to region 20 at the base of the *X* chromosome (MIKLOS *et al.* 1987).

The two original $stoned^{ts}$ alleles (stn^{ts1} and stn^{ts2}) show severe debilitation at elevated temperature (29°), but also exhibit abnormal behavior at permissive temperature (22°). At 22° they are sedentary, rarely move unless agitated, and show little or no geotaxis. The flies do, however, exhibit a strong jump response when subjected to a rapid "light-off" stimulus, resulting in them tumbling over on their backs (KELLY 1983a). This abnormal jump is observed in both stn^{ts1} and stn^{ts2} flies, and is correlated with an increase in the amplitude of the off-transient of the electroretinogram (ERG) in stn^{ts} flies (KELLY 1983a). Comparison of synaptosomal fractions from the heads of stn^{ts} and wild-type flies, showed that a 31-kD protein was overphosphorylated *in vivo*, in extracts from stn^{ts} flies (KELLY 1983b). The *in vivo* phosphorylation of this protein is light-dependent and the altered level of phosphorylation in stn^{ts} flies is thought to be correlated with the abnormalities in the ERG of this mutant (KELLY 1983b).

A third behavioral mutant at the *stoned* locus (stn^{C})

was isolated in a screen for flightless flies (HOMYK 1977; HOMYK and SHEPPARD 1977). Flies carrying the stn^C mutation were shown to exhibit debilitation when mechanically stressed, and originally named *stress sensitive-C*[1]. They were also shown to be inviable when cultured at elevated temperature. Complementation analysis showed that stn^C was an allele of *stoned* (MIKLOS *et al.* 1987), and that all three behavioral mutants were members of a lethal complementation group identified by the lethal mutation *l(1)X-3* (SCHALET and LEFEVRE 1976). In contrast to the stn^{ts} mutants, stn^C flies entirely lack the on- and off-transients of the ERG (HOMYK and PYE 1989).

Many lethal mutations have been isolated and mapped to the *stoned* region of the *X* chromosome (LINDSLEY and ZIMM 1992), including some that were recovered in a screen for putative *P* factor-induced mutations (ZUSMAN, COULTER and GERGEN 1985). Here we report the complementation pattern of the behavioral and lethal mutations at the *stoned* locus, including an analysis of their debilitation, viability and ERG phenotypes.

Although a defect of the visual system has been demonstrated in the stn^{ts} and stn^C mutants (KELLY 1983a,b; HOMYK and PYE 1989), a general defect of the nervous system is likely. As functional relationships may be revealed through studies of genetic interactions among different mutationally altered gene products (eg. GANETZKY 1984; RABINOW and BIRCHLER 1990), we have investigated the genetic interactions of *stoned* mutations with mutations at other loci. At the physiological level, we have already shown that the stn^{ts} mutants can partially suppress the absence of the ERG off-transient in *tan* mutant flies (KELLY 1983a), suggesting a functional link between the *tan* and *stoned* gene products in the visual system. This study describes further specific interactions between *stoned* mutations and mutations at the *dunce* and *shibire* loci, and identifies a novel *Suppressor of stoned* mutation.

MATERIALS AND METHODS

***D. melanogaster* strains:** The Drosophila *stoned* mutants used in this study are shown in Table 1 together with information concerning the origin of the mutations. The *dunce* mutant strains dnc^1, dnc^{M14} (BYERS, DAVIS and KIGER 1981), and dnc^{M14R1} (BELLEN and KIGER 1987), were obtained from J. KIGER. All other mutations used in this study are as described in MIKLOS *et al.* (1987) or LINDSLEY and ZIMM (1992). *Df(1)30A* was originally isolated as a *P* element-induced lethal allele of *l(1)X3* (ZUSMAN, COULTER and GERGEN 1985), and has been recorded as such (LINDSLEY and ZIMM 1992), but has subsequently been shown to be a deficiency of at least 5 adjacent lethal complementation groups surrounding *stoned* (J. ANDREWS, G. L. MIKLOS and L. E. KELLY, unpublished results). In most cases two stocks were maintained for each allele. The lethal *stn* alleles were kept in stocks of males crossed to attached-*X* females both of which carried the modified *Y* chromosome, y^+Ymal^+. This *Y* chromosome carries a duplication for the proximal region of the *X*-chromosome (LINDSLEY and ZIMM 1992), and thus a copy of the stn^+ gene. A second stock of each *stn* lethal was maintained heterozygous with the *X* chromosome balancer *FM6*. For stn^{ts1} and stn^{ts2}, homozygous stocks were kept, each carrying the eye color mutation white (*w*).

Flies were raised on standard cornmeal based fly food, in 250-ml plastic bottles, although some experiments were carried out in 50-ml glass vials. Flies were maintained at a constant temperature of 22° in a 12-hr light/dark cycle.

Complementation analysis: For each pair of alleles the following cross was carried out:

$$stnA/FM6 \times stnB/Y$$

$$\downarrow$$

$$stnA/stnB;\ stnB/FM6;\ stnA/Y;\ FM6/Y$$

where *stnA* and *stnB* represent different alleles of *stoned*, and the *Y* chromosome is replaced with y^+Ymal^+ when *stnB* is a lethal allele. The crosses were carried out in triplicate. The progeny from each cross were scored progressively over a period of 5 weeks from the date the culture was set. Some mutant heterozygotes developed more slowly and this was taken into account when scoring or before discarding cultures.

Electroretinograms: The ERG analysis was performed on females heterozygous for a behavioral and lethal *stoned* allele. For experiments involving *dunce* and *Su(stn)*, male flies were used. All flies tested were between 1 and 5 days old. Due to the nature of the chromosomes used in some of the crosses, some flies were white-eyed and others red-eyed. Removal of the screening pigments in the eye increases the amount of light reaching the photoreceptor cells, and hence the amplitudes of various components of the ERG. The ERG waveforms were, therefore, always compared to a control with the same eye pigmentation. After CO_2 anaesthesia, flies were immobilized in a lateral position, using melted paraffin wax around the legs, wings and proboscis. Tungsten microelectrodes (5 mΩ, A-M Systems, Seattle, Washington) were used for both reference and active electrodes. The reference electrode was inserted in the abdomen, while the active electrode was inserted centrally in the right eye. The light source used was a Volpi Intralux 150H quartz halogen lamp. After a 1-hr period of dark adaptation and recovery from anaesthesia, flies were exposed to a 6-sec pulse of illumination, controlled by a manually operated Leitz camera shutter. The electrode voltage was monitored on an IBM AT computer using the Axon Instruments TL-2 A/D aquisition board and the Axotape Version 1.2 software. Using a known voltage calibration, off-transient amplitudes, in mV, were measured directly from the program, using the voltage difference between the sustained corneal depolarization and the maximum negative off-transient voltage. For each genotype the ERGs of at least five individuals were recorded and in most cases the number was 10. After determining the mean off-transient amplitude for each genotype, a one-way analysis of variance was performed between the classes.

Generation of gynandromorphs: Gynandromorphs were constructed using the unstable ring-*X* chromosome, $R(1)w^{vC}$ (HINTON 1955). $R(1)w^{vC}/In(1)\Delta 49$, *y*, *w*, *lz* females were crossed to *y*, *w*, *spl*, *stn13–120*/y^+Ymal^+ males in the experimental cross, and to *y*, *w*, *spl*/y^+Ymal^+ males in the control cross. The loss of the ring-*X* chromosome could be detected on the cuticle as yellow patches, or on the eye by white patches (HOTTA and BENZER 1972). The mosaic frequency was computed as the ratio of mosaic ring-*X* flies to non-mosaic ring-*X* females, as this definition of mosaic frequency

TABLE 1

***Stoned* alleles**

Allele	Description	Reference
Viable alleles		
stn^{ts2}	EMS induced. 22°–walks slowly, occasionally falls over, flutters wings, can climb and fly. 29°–falls to the bottom of the vial. Incompletely paralyzed, legs kick as they lie on their backs. Mutant phenotype reversible by shifting back to low temperature.	GRIGLIATTI *et al.* (1973)
	EMS induced. 22°–quite active, although not as coordinated as wild type. 29°–unable to climb, walks in a stilted manner.	GRIGLIATTI *et al.* (1973)
stn^{c}	EMS induced. 22°–paralysis following mechanical shock. 29°–die if subjected to mechanical shock.	HOMYK AND SHEPPARD (1977)
Lethal alleles		
Df(1)30A	P-M induced. Lethal	ZUSMAN, COULTER AND GERGEN (1985)
stn^{PH1}	P-M induced. Lethal	ZUSMAN, COULTER AND GERGEN (1985)
stn^{13-120}	Spontaneous. Lethal	SCHALET (1986)
stn^{X3} 1	X-ray induced. Lethal	LIFSCHYTZ AND FALK (1968)
stn^{8P1}	X-ray induced. Semilethal	SCHALET AND SINGER (1971)
stn^{S64}	X-ray induced. Lethal	G. LEFEVRE (unpublished)
stn^{R-1-10}	EMS induced. Lethal	LIFSCHYTZ AND FALK (1969)

allows direct comparison of control and experimental mosaic frequencies (WHITE, DECELLES and ENLOW 1983). The cuticular landmarks on the adult flies (eye, head, individual legs, dorsal and ventral thorax, and abdomen) were scored as being phenotypically wild-type, mutant or mosaic, and the "mutant frequency" for these structures is defined as the number of times the structure was mutant per number of times the structure was scored (WHITE, DECELLES and ENLOW 1983; CAMPOS, GROSSMAN and WHITE 1985). A detailed behavioral record was kept for each mosaic fly.

Anatomical characterization of mosaics: The procedure for preparing histological sections from both wild-type and mosaic animals, was the same as that described by MIKLOS *et al.* (1987).

Protocols for the generation of multiply mutant flies: The *dunce*^{1}-*stoned*^{ts2} and *rutabaga-stoned*^{ts2} double mutants were constructed using marker replacement, such that the presence of both behavioral mutations on the same chromosome could be deduced by the absence of morphological markers.

Similar protocols were used for the generation of the other double and triple mutant combinations. In all cases the double mutants were recovered over the $y^{+}Ymal^{+}$ chromosome when either stn^{ts2} or stn^{C} were involved.

Brood analysis of the double mutant combinations: Three male flies carrying the double mutant combinations in conjunction with a $y^{+}Ymal^{+}$ chromosome were mated to five virgin females carrying an attached-*X* and a normal *Y* chromosome. The adults were left in a vial for 2 days and were then transferred to a fresh vial. This transfer was repeated every second day for a total of 6 days, at which point the adults were discarded. These conditions led to little crowding in individual vials but allowed the assessment of quite large numbers of progeny. The adult progeny were then scored every day as they eclosed, and the number of males and females counted. The behavior of surviving males was also observed.

Mapping the *Suppressor of stoned* mutation: The crosses used to map the *Su(stn)* locus involved using a *sn* (21.0), *lz* (27.7), *v* (33.0), stn^{ts2} chromosome and a *cm* (18.9), *ct* (20), *oc* (23.1), *ptg* (23.2) chromosome. The crosses were performed and scored as follows.

Heterozygous *sn lz v* stn^{ts2}/*Su (stn)*stn^{ts2} females were mated to wild-type females. The male progeny were scored for recombinants within the *sn-lz* interval and then for the *stoned* phenotype, to determine the presence or absence of the *suppressor*.

Similarly heterozygous *cm ct oc ptg v*/*Su(stn)* stn^{ts2} females were mated to wild-type males. The progeny males were first scored for recombinants within the *ct-oc* interval and then scored for their *stoned* phenotype. The recombination frequencies determined from this cross were less accurate than that from the previous cross, due to the possibility of a second, unrecognized, recombination event in the *v* to *stn* interval.

RESULTS

Complementation studies: It had previously been determined that stn^{ts1} and stn^{C} belonged to the *l(1)X-3* complementation group, however, it had also been observed that there were distinct behavioral and viability effects in stn^{ts1}/*(1)X-3* and stn^{C}/*(1)X-3* heterozygote combinations (MIKLOS *et al.* 1987). A number of other lethal mutations are known that fail to complement *l(1)X-3*, and so the complementation of these lethal mutations with the viable *stoned* alleles was investigated. Initial studies showed that there was considerable variation in the level of debilitation between the various stn^{ts}/*stn(lethal)* heterozygotes. As debilitation is difficult to quantify, we used relative viability as a measure of this phenotype.

The relative viabilities of all pairwise heterozygotes of the viable alleles (stn^{ts1}, stn^{ts2} or stn^{C}), heterozygotes of viable alleles with lethal alleles, and stn^{ts2}, stn^{ts1} and stn^{C} homozygotes are summarized in Table 2. Females heterozygous for a lethal allele and either stn^{ts1} or stn^{ts2} survive, although in most cases their viability is

TABLE 2

Percent relative viabilities of *stoned* heterozygotes

	stn^{ts1}	stn^{ts2}	stn^{C}	stn^{8P1}	stn^{13-120}	stn^{S64}	stn^{X3}	stn^{PH1}	stn^{R-9-10}	*Df(1)20)*
stn^{ts1}	55	64	82	43	63	56	35	21	16	18
stn^{ts2}		62	86	79	66	65	42	31	22	21
stn^{C}			64	55	48	58	54	54	58	56

The methods used to determine the relative viabilities of the various heterozygous *stoned* flies are described in MATERIALS AND METHODS.

reduced in comparison with the equivalent homozygous strain. It was noted that *stoned* mutant flies pupate later than *stoned*$^{+}$ flies, and experience greater difficulty in emerging from their pupal cases. Often, after they have emerged, debilitated flies become mired in the medium. This is especially true of the $stn^{ts1}/Df(1)30A$ and $stn^{ts2}/Df(1)30A$ heterozygotes, which survive, but show extremely low relative viabilities.

The stn^{ts} alleles, in heterozygous combination with stn^{PH1}, stn^{R9-10} and *Df(1)30A* lead to flies with the lowest relative viabilities. There are no significant differences in the viability values for these heterozygotes, suggesting that stn^{PH1} and stn^{R9-10} alleles may represent null alleles of the *stoned* locus. The stn^{ts}/stn^{13-120}, stn^{ts}/stn^{S64} and stn^{ts}/stn^{8P1} heterozygotes have viabilities that are not significantly different from stn^{ts} homozygotes. This implies that although stn^{13-120}, stn^{S64} and stn^{8P1} are homozygous lethal, they are not behaving as complete nulls. This is certainly the case for stn^{8P1}, which, although originally isolated as a lethal (SCHALET and SINGER 1971), has subsequently been shown to be "semilethal," with stn^{8P1} flies occasionally surviving to adulthood (MIKLOS *et al.* 1987, and this work). The stn^{X3}/stn^{ts} heterozygotes are intermediate between these two groups of mutants.

The behavioral analyses of the heterozygotes largely reflect the findings of the viability study. The $Df(1)30A/stn^{ts}$, stn^{PH1}/stn^{ts} and stn^{R9-10}/stn^{ts} heterozygotes are extremely inactive and uncoordinated, whereas the others are more like stn^{ts} homozygotes.

The mean viabilities of the stn^{C} heterozygotes (Table 2) do not vary greatly, and lie in the 50–60% range. The only exceptions are stn^{ts1}/stn^{C} and stn^{ts2}/stn^{C}. These heterozygotes have a mean relative viability that is significantly higher than that of the other stn^{C} heterozygotes and stn^{ts1}, stn^{ts2} and stn^{C} homozygotes. This suggests that there is intra-allelic complementation between the stn^{ts2} and stn^{C} alleles. This intra-allelic complementation is also reflected in the behavior of these flies. The stn^{C} allele appears to entirely complement the stn^{ts2} uncoordinated phenotype, although the stn^{ts2} allele does not complement the stn^{C} stress-sensitive behavior.

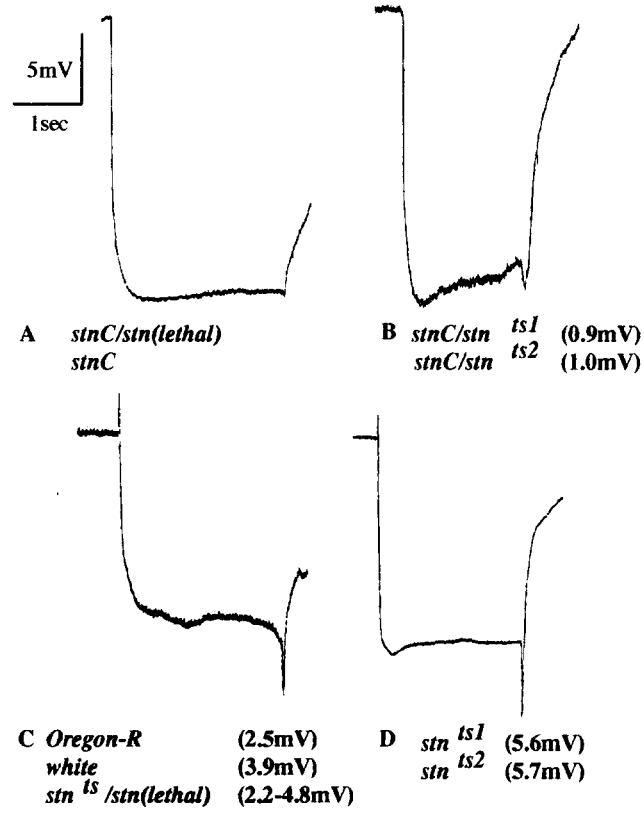

FIGURE 1.—Typical ERG recordings from various mutant *stoned* flies. These fall into four classes A, B, C and D, which form a graded series with respect to the amplitude of the off-transient, from a zero value (A) through an intermediate value (B) and wild-type (C), to the augmented value seen in stn^{ts} homozygotes (D). The numbers shown in brackets represent the mean values of the off-transients for each of the genotypes. The greatest variation is seen in group C, due to the presence of white eyed and red eyed heterozygotes in this class. One way ANOVA with $N \geq 5$, was carried out to distinguish between the various classes (not shown).

The stn^{ts} and stn^{C} mutants also show distinct ERG phenotypes. The stn^{ts} mutants show an increased amplitude of off-transient, while stn^{C} exhibits the loss of both transient responses (KELLY 1983a; HOMYK and PYE 1989). The ERG phenotypes of the heterozygotes were investigated as part of the complementation analysis, and found to comprise three distinct classes (Figure 1). Flies that were heterozygous for a lethal allele and stn^{C} displayed either a small or no off-

TABLE 3

A. Mosaic frequencies for experimental and control mosaics

	Inferred genotype	No.	Mosaic frequency[a]
Control cross			
Non-mosaic flies	*(R(1) wv^c/y,w,spl)*	87	1.23
Mosaic flies[b]	*(R(1) wv^c: O/y,w,spl)*	107	
Experimental cross			
Non-mosaic flies	*(R(1) wv^c/y,w,spl,stn^{13-120})*	266	0.51
Mosaic flies	*(R(1) wv^c: O/y,w,spl,stn^{13-120})*	135	

B. Average mutant frequencies for landmarks on adult mosaics

Mosaics	Head	Dorsal thorax	Ventral thorax	Abdomen
y,w,spl,stn^+	0.35	0.40	0.40	0.43
y,w,spl,stn^{13-120}	0.09	0.18	0.12	0.23

[a] Mosaic frequency is the ratio of mosaic: non-mosaic adult flies.
[b] Mosaic flies also include X/O males.

transient, with $stn^C/stn^{R9\text{-}10}$, stn^C/stn^{PH1} and stn^C/stn^{S64} flies showing small off-transients (<1 mV). When the lethals *stoned* alleles were placed in heterozygous combination with *w* stn^{ts1} and *w* stn^{ts2}, the amplitude of the off-transient was found to be at a level intermediate between stn^{ts2} and stn^C homozygotes (Figure 1). This value did not differ significantly from the white or red eyed controls in a one-way analysis of variance. Flies heterozygous for stn^C and either stn^{ts1} or stn^{ts2} constitute a third class. The off-transient amplitudes are significantly lower than the white control, and lower than the stn^{ts}/*stn(lethal)* class.

Mosaic analysis of *stoned*: Although the phenotypes of the viable *stoned* alleles suggest that the *stoned* gene product has a role in nervous system function, it is possible that the gene has functions in other tissues. An organismal lethal mutation, such as the *stoned* lethal mutations, may or may not be essential to the survival of all cells (RIPOLL and GARCIA-BELLIDO 1973). If the *stoned* gene function is essential to all cells, a gynandromorph with large cuticular mutant patches would be unlikely to survive. We investigated the possible role of *stoned* in general cellular functions by using the spontaneously induced lethal allele $stn^{13\text{-}120}$ to determine whether cellular clones hemizygous for this *stoned* lethal allele could survive in genetic mosaics. The survival of some $stn^{13\text{-}120}$ /0 mosaics suggests that wild-type levels of *stoned* gene product is not required in all cell types.

The frequencies of mosaics in both the *stn* and control crosses are shown in Table 3A. Males of the genotype *y, w, spl,* $stn^{13\text{-}120}$/0 (X/O), and approximately 58% of the expected $stn^{13\text{-}120}$ mosaic progeny failed to survive. If the lethality associated with the $stn^{13\text{-}120}$ mutation is tissue specific, then the surviving $stn^{13\text{-}120}$ mosaics should have a nonrandom distribution of mutant tissue biased against those tissues in which the *stoned* gene product is expressed. Table 3B gives the average mutant frequencies for experimental and control mosaic populations. Whereas the average mutant frequencies for the control cross were relatively uniform (average of 0.40 for all structures), the frequencies were lowered for all structures in the mosaics, with the most significant decrease in head tissue (Table 3B). The stn^{13};$^{-120}$ mosaics consisted of individuals with small patches of mutant tissue, which indicates that flies with larger mutant patches could not survive. No $stn^{13\text{-}120}$ mosaic had a completely mutant head or thorax, and, with a single exception, no mosaic was mutant for more than half the head cuticle. Presumably a fly with an entirely mutant head would be inviable. The absence of mosaics with an entirely mutant thorax indicates that this condition is also lethal. As the size of the mutant patch increased, so also did the degree of aberrant behavior. Mosaics with mutant tissue on the ventral thorax, almost invariably had problems with leg coordination, posture and ability to climb. In mosaics with mutant head cuticle only, behavior was normal if the mutant patch was very small. However, as the size of the mutant patch increased, the fly's behavior became more sedentary. Head-only mosaics always retained normal coordination. Mosaics with mutant abdominal tissue were for the most part normal, however an occasional abdominal mosaic showed an affected meso-thoracic leg, presumably due to internal mosaicism that was not defined by the cuticular layer.

From this analysis it appears that the *stoned* gene product is required in the head for normal active behavior and in the thorax for motor coordination and posture, and reinforces the idea that *stoned* is a neurological gene.

Anatomical analysis of mosaics: The observations of $stn^{13\text{-}120}$ mosaics, indicated that *stoned* lethality was most closely associated with mutant head tissue. To examine the possibility that the underlying neural tissue in $stn^{13\text{-}120}$ head mosaics might exhibit abnormal morphology, flies with one mutant eye (or mosaic eye)

and one wild-type eye were sectioned, and stained. Examination of 11 such mosaic individuals gave no indication of any significant alterations in gross brain morphology.

Modifiers of *stoned*

***dnc* mutations enhance the *stn^{ts}* phenotype:** Mutants with similar phenotype may be functionally related, such that the phenotype of the double mutant combination may be more severe than either mutant alone (GANETZKY 1984). To identify possible modifiers of *stoned*, mutations that affect loci with biochemical similarities to the *stoned* mutants were considered. The *stn^{ts}* mutants have been shown to have altered *in vivo* levels of cyclic-AMP-dependent phosphorylation of a synaptosomal protein (KELLY 1983b), and hence mutations that alter cAMP metabolism might be expected to interact with *stoned* mutants. One such locus is *dunce* which encodes an isoform of cAMP phosphodiesterase (PDE II) (BYERS, DAVIS and KIGER 1981). Mutations at the *dunce* locus result in elevated cAMP levels in adult flies, but complete null alleles at this locus are viable. Mutations at a second locus, *rutabaga*, which encodes one form of adenylate cyclase (LIVINGSTONE, SZIBER and QUINN 1984; LIVINGSTONE 1985), might be expected to lower intracellular cAMP levels, but are also viable.

To construct *dnc-stn* double mutants, the *dnc^{1}* and *dnc^{M14}* alleles were used. The *dnc^{1}* allele is believed to be a hypomorph, whereas the *dnc^{M14}* mutant allele is a null mutation (DAVIS and KIGER 1981). The cAMP levels in these mutant flies have been shown to be, respectively, 1.4 and 6 times greater than wild type (BYERS, DAVIS and KIGER 1981). In terms of general fitness, both mutants appear to be normal (SALZ, DAVIS and KIGER 1982).

Despite the considerable genetic distance between the two loci, initial attempts to isolate the recombinant *dnc^{1}-stn^{ts2}* double mutant were unsuccessful. The double mutant chromosome could be isolated in males that carried a *stn^{+}* duplication on the *y^{+}Ymal^{+}* chromosome. These males were indistinguishable from *dnc^{1}* mutant flies. However, when these *dnc^{1}-stn^{ts2}/ y^{+}Ymal^{+}* males were outcrossed to attached-*X* females carrying a normal *Y* chromosome, the resulting *dnc^{1}-stn^{ts2}/Y* male progeny failed to eclose. Most of the males died within their pupal cases, and although a few did manage to eclose, they immediately fell to the bottom of the vial and became mired in the medium. When recently eclosed males were rescued and placed in an empty vial for observation, it was found that they were totally uncoordinated and were unable to walk or right themselves. This phenotype is similar to the phenotype of homozygous *stn^{8P1}* survivors and to the most severe of the mosaic individuals described above. A similar result was found for *dnc^{M14}-stn^{ts2}* double mutants but with fewer males managing to eclose. As a control the *dnc^{M14}* revertant, *dnc^{M14R1}* (BELLEN and KIGER 1987) was also used to construct the double mutant. The phenotype of these flies was similar to that of *stn^{ts2}* flies. The data from a brood analysis of the various *dunce-stoned* mutants strains, mated to attached-*X* females with a normal *Y* chromosome, are shown in Figure 2.

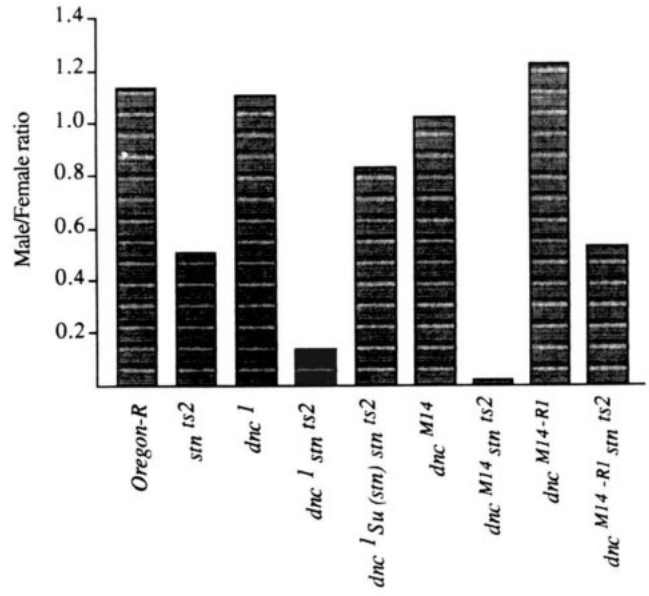

FIGURE 2.—Brood analysis showing the effects of the *dunce* mutations on the viability of the *stn^{ts2}* mutant. The crosses were set up as described (MATERIALS AND METHODS) and the ratio of surviving mutant males to attached-*X* females is plotted for each genotype.

The *dnc^{1}-stn^{C}* double mutant was also constructed, using the same protocol as that given for *dnc^{1}-stn^{ts2}*. This mutant combination was found to be viable. The *dnc^{1}-stn^{C}* mutant males show no greater debilitation or stress-sensitivity than *stn^{C}* males. The failure to observe any interaction between *dnc^{1}* and the *stn^{C}* allele suggests that the lethal interaction is specific to the *stn^{ts2}* allele. This specificity of interaction is further demonstrated by the construction of the *dnc^{1}-para^{ts1}* and *dnc^{1}-shi^{ts1}* double mutants. Neither of these combinations resulted in a more severe phenotype than either of the paralytic mutations on their own. Nor did the *rut-stn^{ts2}*, *para^{ts1}-stn^{ts2}*, and *Sh^{102}-stn^{ts2}* double mutants show any greater effect on viability than the *stn^{ts2}* mutation on its own.

An analysis of the ERGs of *dnc-stn^{ts2}* double mutants was carried out, but only survivors could be scored. By definition, these flies constitute a subpopulation of the genotype, and hence the data were not amenable to statistical analysis. Many flies did not survive the immobilization procedure. However, ERG traces from four flies which did survive, had off-transient amplitudes greater than 7 mV. This value is almost double the *dunce* mean of 3.76 mV, and indeed greater than the *w stn^{ts2}* mean (5.7 mV), and this

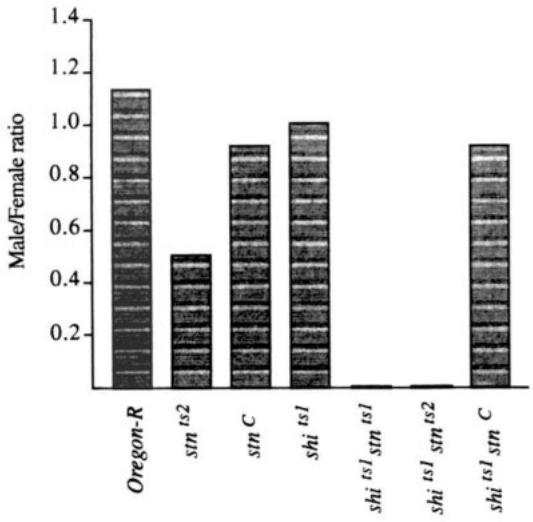

FIGURE 3.—Brood analysis showing the effects of the shi^{ts1} mutation on the viability of *stoned* mutants using the procedure as outlined in the legend to Figure 2.

despite the presence of red eye pigment in the *dnc*-stn^{ts2} double mutant.

The shi^{ts1} mutation also enhances the stn^{ts} phenotype: As a further test of the specificity of the interaction between stn^{ts2} and *dnc*, the shi^{ts1}-stn^{ts2} double mutants were constructed. Again the double mutant recombinants were isolated in males carrying the y^+Ymal^+ chromosome. When these shi^{ts1}, stn^{ts2}/y^+Ymal^+ males were mated to attached-*X* females with a normal *Y* chromosome, no male progeny were obtained (Figure 3). In contrast to the *dnc*-stn^{ts2} interaction, the lethality expressed in these double mutants is complete. However like the interaction with *dnc*, stn^C showed no interaction with shi^{ts1} (Figure 3). Again, the specificity of this interaction suggests that the action of the *shibire* and *stoned* gene products must somehow overlap.

A *Suppressor of stoned* (*Su(stn)*): While both *dnc* and shi^{ts1} enhance the phenotype of stn^{ts2} flies, a third locus, *Suppressor of stoned* (*Su(stn)*), suppresses the stn^{ts2} phenotype. The *Su(stn)* was identified in a stn^{ts2} stock that had been kept homozygous in laboratory culture for more than 10 years. This stn^{ts2} strain no longer exhibited sedentary behavior nor did it exhibit temperature-sensitive debilitation. On outcrossing the suppressed stn^{ts2} strain with a multiply marked *X* chromosome (*w ct f*), the stn^{ts2} mutant phenotype could be recovered among *X* chromosome recombinants, indicating that a major *X*-linked modifier was present. An analysis of the behavior of the F_2 progeny showed that the *suppressor* variant was located between the markers *ct* and *f*, but close to *ct*, such that most *w ct* male recombinants exhibited the *stoned* phenotype.

Using the phenotypic suppression of stn^{ts2} by *Su(stn)* as the criterion for the presence of *Su(stn)*, the suppressor mutation has been more precisely mapped. Two multiply marked *X* chromosomes were used; a *sn lz v* stn^{ts2} chromosome, and a *m ct oc ptg v* chromosome as described in MATERIALS AND METHODS. The results using the *sn lz v* stn^{ts2} chromosome indicate that the suppressor locus is located within the 6.7 map units between *sn* and *lz*, and closer to *sn* at approximately 22 map units. This location was confirmed using the results from the cross with *cm*, *ct*, *oc*, *ptg*, *v*, which placed the *suppressor* between *ct* (20 map units) and *oc* (23.1 map units). This corresponds to the cytological region 7D–8A.

Having mapped the *Su(stn)*, it was then possible to identify *Su(stn)*-stn^+ recombinants. In the absence of a mutant *stoned* allele, the *Su(stn)* variant is phenotypically wild type in both behavior and viability. Phenotypically, the presence of the *Su(stn)* could be ascertained only in combination with *stn* mutations. To determine whether the *Su(stn)* allele was dominant or recessive to the wild-type allele, the relative viabilities of stn^{ts2} homozygous females carrying two, one or no copies of the *Su(stn)* allele was examined. Using relative viability as an indicator, it is clear that stn^{ts2} females carrying two copies of the *Su(stn)* mutant allele are equivalent to wild type (100%), whereas the females without a *Su(stn)* allele have lower relative viabilities (62%). A single copy of the *Su(stn)* in female stn^{ts2} homozygous flies results in intermediate viabilities (86%). The partial dominance of the suppressor also extends to the behavioral phenotype. Homozygous *stoned* flies carrying one copy of the suppressor are not as active as those with two, but more active than those with none. It appears then that the *Su(stn)* mutant allele behaves in a semidominant manner with respect to its wild-type allele.

The ERGs of *w* stn^{ts2} and *w Su(stn)* stn^{ts2} flies were also compared. The *w* stn^{ts1} and *w* stn^{ts2} off-transient amplitudes are similar, and both significantly higher than the white-eyed control (Figure 1). When *Su(stn)* was crossed on to the *w* stn^{ts2} chromosome, the off-transient amplitude of these males was reduced to the statistical equivalent of the control (Figure 4). Thus it appears that *Su(stn)* acts to reduce the off-transient amplitude of stn^{ts2} flies to wild-type levels.

The effects of *Su(stn)* on other *stoned* alleles: The *Su(stn)* was placed in cis to stn^{ts1}, stn^C, stn^{8P1}, stn^{S64}, $stn^{13\text{-}120}$, $stn^{X\text{-}3}$, and $stn^{R9\text{-}10}$. Where a *stoned* lethal allele was being investigated recombinant males were rescued over the y^+Ymal^+ chromosome. These *Su(stn)*, *stn* males were then crossed to attached-*X* females carrying a normal *Y* chromosome and the number of female and male progeny was noted. Both the stn^C and stn^{ts1} males are relatively more viable when the *Su(stn)* is present, with the ratio of stn^{ts1} and stn^C males to attached-*X* females reaching wild-type levels in the presence of the *Su(stn)* allele. Males carrying the lethal alleles stn^{S64}, stn^{X3}, $stn^{13\text{-}120}$ and $stn^{R9\text{-}10}$ did not survive even in the presence of the suppressor. However,

FIGURE 4.—The effects of the *Su(stn)* on the off-transient amplitude in stn^{ts} flies, showing the reduced amplitude of off-transient in the presence of *Su(stn)*.

whereas no stn^{8P1} males survived under the conditions of this experiment, *Su(stn)* stn^{8P1} males did survive (male to attached-*X* female ratio of 5%), indicating that the *Su(stn)* partially suppresses this semilethal mutation. Not only was the viability of the stn^{ts1}, stn^{C}, and stn^{8P1} flies enhanced by the presence of the suppressor, but their behavior more closely resembled wild type. In addition, females of the genotype *Su(stn)* $stn^{X\text{-}3}/stn^{ts2}$, were indistinguishable from females that were *Su(stn)* $stn^{ts2}/stn^{X\text{-}3}$, leading to the conclusion that suppression was independent of the *cis/trans* configuration of the suppressor with respect to the *stoned* mutant alleles.

The triple mutant dnc^{1}-*Su(stn)*-stn^{ts2} was also constructed. The viabilities and behavior of the triple mutant males with a normal *Y* chromosome, were wild-type, and could not be distinguished from *Su(stn)* stn^{ts2} individuals (Figure 2). The suppressor was therefore able to suppress the dnc^{1}-stn^{ts2} lethal interaction.

Although far from exhaustive, our search for other mutations that might be suppressible by the *Su(stn)*, including alleles at the *dunce*, *paralytic*, *Shaker* and *shibire* loci, has uncovered none.

DISCUSSION

The various mutations at the *stoned* locus exhibit a spectrum of phenotypes. The behavioral and neurophysiological phenotypes associated with the stn^{ts} and stn^{C} mutations, and the severe debilitation shown by surviving stn^{8P1} males are all consistent with the gene being involved in nervous system function. A neurological function for the *stoned* gene product is further suggested by the $stn^{13\text{-}120}$ mosaic analysis. The fact that both behavior and viability are more greatly affected in mosaics where the anterio-ventral regions are mutant, indicates that the *stoned* gene product is essential for the normal functioning of cells derived from the anterio-ventral blastoderm region. This region gives rise to neural, muscular and alimentary tissues. A defect in both the neural and muscular tissues could give rise to the *stoned* mutant phenotype. It is probable, however, that the primary focus of the *stoned* gene is in neural tissue, as the difference in mutant frequencies between control and $stn^{13\text{-}120}$ mosaics, and the distribution of $stn^{13\text{-}120}$ mutant tissue, most closely parallels that found when other lethal nervous system mutants are used to generate mosaics (GANETZKY 1984; WHITE DECELLES and ENLOW 1983). Behavioral analysis of the mosaics indicated that the sedentary nature of *stoned* mutant flies is related to head tissue, while the uncoordinated behavior is associated with mutant thoracic tissue, particularly in the legs and ventral structures. It also seems likely that the lethality of $stn^{13\text{-}120}$ flies results from a critical reduction in levels of *stoned* gene-product in the nervous system. The $stn^{13\text{-}120}$ mutation results from an insertion event (J. ANDREWS and L. E. KELLY, unpublished data) but is not a null mutation as it does not behave in a manner equivalent to the deletion mutant *Df(1)30A* in the complementation experiments. We cannot, therefore, rule out the possibility that the *stoned* gene product is also active in non-neural tissues, albeit at a subcritical level.

The anatomical observations of the mosaics suggest that, while normal levels of the *stoned* gene product are essential for nervous system function, a severe reduction in *stoned* activity has no effect on the gross development of the nervous system.

The complementation data give some clues as to the nature of the nonlethal *stoned* alleles. In heterozygous combination with the stn^{ts} alleles, there is a gradation of severity among the *stoned* lethal alleles, with some behaving in a manner similar to the deficiency, while others survive to eclosion but show severe lack of coordination and premature death. This suggests that a deficiency of *stoned* gene product leads to uncoordinated motor output, and that the stn^{ts} alleles are behaving as hypomorphs. Similarly, the lack of ERG transients in the stn^{C}/*lethal* heterozygotes and the reduction in the transient amplitudes in the stn^{ts}/*lethal* heterozygotes would suggest that the absence of the transients is also a null phenotype, and that stn^{C} is also a hypomorph. Yet in other respects, the phenotypes of stn^{C} and stn^{ts} are not those of null mutations. The ERG off-transients in stn^{ts} flies are larger than normal, whereas in stn^{ts}/stn^{C} flies the off transient is less than that expected if stn^{C} were equivalent to a deficiency. This suggests that, at the level of the ERG

off-transient, the stn^{ts} mutant behavior is that of a hypermorph while stn^{C} behaves as an antimorph. Given the contrary behavior of these two alleles, it is not surprising that much of the individual effects of the two mutations are found to nullify each other in the stn^{ts}/stn^{C} heterozygote, which shows increased viability and coordination although retaining some measure of stress sensitivity. The partial complementation between these two alleles suggests that the *stoned* gene product is multifunctional, with the stn^{C} mutation affecting one function while the stn^{ts} mutation affects a second.

A nervous system function for the *stoned* gene product is also supported by the results of the *dunce-stoned* double mutant analysis. The biochemical analysis of *stoned* and *dunce* has indicated a role for both gene products in the pathway of protein phosphorylation (KELLY 1983b; DÉVAY *et al.* 1984, 1986; DÉVAY and FREIDRICH 1987; BUXBAUM and DUDAI 1989). Two mutants, if functionally related, may interact phenotypically in a manner more extreme than the simple addition of the two phenotypes. This appears to be the case with the $dnc\text{-}stn^{ts2}$ double mutant, with the phenotype of the double mutant combinations being more severe with the stronger *dunce* allele dnc^{M14}. The failure to observe any interaction between stn^{ts2} and *rut*, $para^{ts1}$ or Sh^{102}, implies that addition of a second neurological mutation, and even one that is defective in cAMP metabolism, is not in itself sufficient to reduce the viability of stn^{ts2}. It appears more likely, therefore, that the $dnc^{1}\text{-}stn^{ts2}$ interaction is due to an underlying functional relatedness rather than mutational overload. The phenotype of stn^{ts2} in combination with the $dnc^{M14\text{-}R1}$ revertant also excludes the possibility that the phenotypic interaction with *stoned* is due to some other cryptic mutation in the *dunce* strains rather than the *dunce* locus itself.

The severely uncoordinated phenotype observed in the $dnc\text{-}stn^{ts2}$ double mutants is not inconsistent with the known site of action of *dunce*. The *dunce* gene is known to be involved in the conditioning of leg-positioning (BOOKER and QUINN 1981), and more recently has been shown to increase the fine ramifications of a mechanosensory neuron (CORFAS and DUDAI 1991). Presumably therefore, *dunce* gene product is somehow involved in the modification of the mechanosensory/motor circuit that controls leg position. The lack of motor coordination in stn^{ts} mutants also suggests a defect in this circuit, and so the action of the *dunce* mutations to enhance the stn^{ts} defect, is not surprising. However, the failure of *dunce* mutations to show any effect with stn^{C} emphasizes the difference between the stn^{ts} and stn^{C} alleles. While it is probable that the *dunce* and *stoned* genes interact at the gene-product level, it cannot be ruled out that *stoned* transcription is regulated by cAMP, as has been found for other genes (CHERRY *et al.* 1988; YAMAMOTO *et al.* 1988).

The observation that the $dnc^{1}\text{-}stn^{ts2}$ double mutant has an even greater off-transient amplitude than stn^{ts2} on its own can be explained in one of two ways. It is possible that the increase levels of cAMP caused by the the dnc^{1} mutation, amplifies the deleterious effect of the stn^{ts2} mutation on the ERG off-transient. However it is also possible that the large off-transients of the double mutant flies represents one extreme of the normal distribution of stn^{ts2} ERG phenotypes, and that only those flies that express the stn^{ts2} phenotype within this range, survive the presence of the dnc^{1} mutation. The latter possibility seems the more likely as *dunce* mutations have no effect on the ERG, and recent studies have shown that the *dunce* gene product is not expressed in the visual system of the fly (NIGHORN, HEALY and DAVIS 1991).

The discovery of the lethal interaction between the stn^{ts} and shi^{ts1} mutations was serendipitous. This combination was meant to act as a control for the *dunce-stoned* interaction. Although it is possible that the effect of these two mutations together in the same fly may merely be an additive effect of two neurological mutations, the failure to obtain any $shi^{ts1}\text{-}stn^{ts2}$ males under non-crowding conditions, makes this possibility unlikely, as does the failure to see any interaction between stn^{C} and shi^{ts1}. Both *shibire* and *stoned* exhibit neurological phenotypes. Both affect the production of the transients of the ERG (KELLY and SUZUKI 1974; KELLY 1983a; HOMYK and PYE 1989). In *shibire* flies the temperature-sensitive paralysis phenotype, and presumably the loss of the ERG transients, is due to a failure to recycle pre-synaptic membrane (POODRY and EDGAR 1979; KOSADA and IKEDA 1983; MASUR, KIM and WU 1990). If the *stoned* gene is also involved in regulating processes at presynaptic terminals, then the interaction between mutations in these two genes may be readily explained.

The *Su(stn)* mutation is curious in its behavior. Its partial dominance and its ability to suppress both the stn^{ts} and stn^{C} mutations almost entirely, and the stn^{8P1} mutation partially, does not allow a simple interpretation of its mode of action. Presumably, *Su(stn)* could act in a manner similar to other known suppressors such as *Suppressor of Hairy wing* (*Su Hw*) to alleviate the effects of transposable element insertions (MODOLELL, BENDER and MESELSON 1983; RUTLEDGE *et al.* 1988). In keeping with this interpretation is the absence of any phenotype associated with *Su(stn)* on its own. However, the findings that *Su(stn)* is only partially dominant detracts from this explanation, and a second possibility that might be considered would involve the *Su(stn)* variant acting to alleviate the deleterious effects of the *stoned* mutations at the level of the *stoned* gene product.

The results described here indicate an essential role for the *stoned* gene product in nervous system function in two areas, the visual system and in motor coordination. It has been possible, using the stn^{ts} and stn^{C} alleles, to genetically differentiate between the roles played by the *stoned* gene in these processes. The data also suggest that the *stoned* gene product interacts, directly or indirectly, with the cAMP second messenger system, and the endocytotic pathway via dynamin. The previously described interaction with *tan* (KELLY 1983b) also implies an interaction of *stoned* with the metabolism of biogenic amines, as it has been shown that the *tan* locus encodes a β-alanyl dopamine hydrolase (WRIGHT 1987). To understand all of these interactions at the mechanistic level, including those with the suppressor, will require the cloning of the *stoned* gene and the identification of its product.

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