Diverse Expression and Distribution of *Shaker* Potassium Channels during the Development of the *Drosophila* Nervous System

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The spatio-temporal expression of *Shaker* (*Sh*) potassium channels (*Kch*) in the developing and adult nervous system of *Drosophila* has been studied at the molecular and histological level using specific antisera. *Sh Kch* are distributed in most regions of the nervous system, but their expression is restricted to only certain populations of cells. *Sh Kch* have been found in the following three locations: in synaptic areas of neuropile, in axonal fiber tracks, and in a small number of neuronal cell bodies. This wide subcellular localization, together with a diverse distribution, implicates *Sh Kch* in multiple neuronal functions.

Experiments performed with *Sh* mutants that specifically eliminate a few of the *Sh Kch* splice variants clearly demonstrate an abundant differential expression and usage of the

wide repertoire of *Sh* isoforms, but they do not support the idea of extensive segregation of these isoforms among different populations of neurons. *Sh Kch* are predominantly expressed at late stages of postembryonic development and adulthood. Strikingly, wide changes in the repertoire of *Sh* splice isoforms occur some time after the architecture of the nervous system is complete, indicating that the expression of *Sh Kch* contributes to the final refinements of neuronal differentiation. These late changes in the expression and distribution of *Sh Kch* seem to correlate with activity patterns suggesting that *Sh Kch* may be involved in adaptive mechanisms of excitability.

Key words: potassium channels; *Shaker*; *Drosophila*; *K-channel* expression; *K-channel* localization; *K-channel* distribution

In the last decade, the combination of electrophysiology and molecular biology has yielded great advances in the understanding of the molecular diversity and the structure–function relationship of ionic channels (Hille, 1992). In spite of this, the specific function of each particular channel transcript is not well understood. This is often because of the lack of correlation between *in vitro*-expressed cloned channels and *in vivo* functions.

Potassium channels (*Kch*) are probably the most diverse class among ion channels (Rudy, 1988; Miller, 1991; Rudy et al., 1991; Jan and Jan, 1992, 1994; Pongs, 1992; Salkoff et al., 1992). Multiple *Kch* proteins, having a common central region and different N and C termini, are produced from the *Shaker* (*Sh*) transcription unit by differential and alternative splicing (Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988). Four *Sh* subunits assemble to form a functional channel (MacKinnon, 1991) with different electrical properties depending on the subunit composition (Iverson and Rudy, 1990; Stocker M et al., 1990). Thus, the regulation of both transcription and splicing of *Sh* may determine the intrinsic electrical properties of *Drosophila* neurons.

The study of channels in the nervous system has usually relied on electrical recording from cell bodies. Unfortunately, this is difficult to apply to the study of channels located in dendrites, axons, synapses, and so forth. In addition, very little is known

about the mechanism of subcellular distribution and the subunit association of channel proteins. An understanding of the role played by the broad repertoire of ion channels expressed in the nervous system, therefore, would require the study of channel distribution in nerve cell populations and their subcellular localization within the complex neuronal architecture. Immunohistochemical techniques, using high-affinity antibodies against specific ion channels, provide the most convenient method for approaching this question. The possibility of carrying this immunocytological study to *Drosophila* presents the additional advantage of using the numerous existing mutations to genetically dissect regulatory pathways.

Surprisingly, there have been only two reports in the literature on the immunolocalization of K^+ -channels in *Drosophila* (Schwarz et al., 1990; Becker et al., 1995). The shortage of experimental results in such an active field of research probably reflects the lack of high-affinity antisera for elucidating this important matter.

We have recently raised an antiserum against *Sh Kch* that specifically labels multiple *Sh* proteins in extracts of nervous tissue at different developmental stages (Rogero and Tejedor, 1995).

As an additional step toward unraveling the roles played by this family of ion channels, the present study is the first to examine the tissular distribution and cellular localization of *Sh Kch* during the development of the nervous system of *Drosophila*. Thus, the aim of this work is to understand how the wide repertoire of *Kch* subunit variants generated by the *Sh* transcription unit is spatially and temporally expressed.

MATERIALS AND METHODS

Fly stocks, culture, and harvesting. *Drosophila melanogaster* stocks were grown in standard medium at 25°C with 12 hr dark/light cycles. The *Canton-S* (*CS*) strain was used as a wild-type (*wt*) control, although no

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differences were found with other common *wt* strains such as *Berlin*. *w* Mutants, which lack eye pigment, were used as *wt* control flies for most experiments examining expression in the retina. Males of the mutant strains *B55^D/W32^P/C(1)M3/0* (*Df*); *T(X;Y)W32/FM7a/Y* (*W32*); *T(X,Y)B55/y w f XX* (*B55*); *ShE62/C(1)M3; w sev*; and *sc10.1* were also used. Staging of pupae was according to Bainbridge and Bownes (1981).

Antisera production and purification. Production and affinity purification of the antiserum α -ShHX1 was performed as reported previously (Rogero and Tejedor, 1995). In brief, rabbit antiserum was raised against a recombinant fusion protein containing a sequence stretch of the central core region that is common to all *Sh* splice variants. Antisera were affinity-purified and their specificity tested by comparing Western blot (WB) analysis of total proteins from *CS* and *Sh*-deficient (*Df*) flies, which lack genomic DNA from the *Sh* coding region (Pongs et al., 1988). The lack of any labeled band in the *Df* immunoblot clearly demonstrates the high specificity of these antisera (Rogero and Tejedor, 1995).

Electrophoresis, WB, and immunoblots. Regular monodimensional SDS-PAGE was used according to Laemmli (1970) and modified by Rogero and Tejedor (1995). Tissue samples (whole flies, pupae, and so forth) were homogenized at 80°C in 50 mM Tris, 25 mM KCl, 2 mM EDTA, 0.3 M sucrose, and 2% SDS, pH 7.4, with a microglass potter. Samples were then boiled for 5 min in SDS-PAGE sample buffer and 20 mM DTT. Gels were usually loaded with 12 μ l of each sample. WB were done by electrotransferring the proteins of the gel to nitrocellulose membranes under standard conditions. Immunoblots were performed as described previously (Rogero and Tejedor, 1995). The chemiluminescent ECL (Amersham, Arlington Heights, IL) method was used for the final detection of antibody-binding.

Histology and immunohistology. Mature third instar larvae were dissected in cold Ringer's solution, and brains were fixed at 0°C in nonalcoholic Bouin's fixative for 30–60 min. After immunostaining, brains were dehydrated with alcohol and embedded in SPURR for microsectioning.

Ether-anesthetized flies with no previous fixation were rapidly embedded in Tissue-Tek (Miles, Elkhart, IN), appropriately oriented, and frozen instantaneously in liquid nitrogen. Frontal, horizontal, and sagittal 12 μ M sections were made in a microtome cryostat and fixed in nonalcoholic Bouin's at 4–8°C for 1 hr. Tangential cryostat sections from retina were sequentially fixed with paraformaldehyde (10 min) and nonalcoholic Bouin's fixative (30 min) at 4–8°C.

Immunostaining of whole-mount and cryostat sections was performed with an overnight incubation at 8–10°C with affinity-purified α -ShHX1 antiserum (Rogero and Tejedor, 1995). A biotin-coupled anti-rabbit IgG and an avidin–biotin–HRP complex were used sequentially under standard conditions to produce the final reaction of DAB.

To see the cellular structure of the retina, proboscides and air sacks were quickly removed from heads in ice-cold 4% paraformaldehyde. Heads were sequentially fixed with paraformaldehyde and Bouin's fixative as before, and embedded in SPURR. Tangential 4 μ M sections were made of the retina and stained with 0.02 methylene blue/0.02% toluidine blue in 0.1% borax.

Toluidine blue counterstaining of either plastic or cryostat sections was performed at 40°C with 0.05% toluidine blue in 0.1% borax. Under these conditions, only cellular structures lacking DAB-precipitate are stained blue.

RESULTS

Distribution and localization of *Sh* Kch in the CNS

In a previous immunochemical study (Rogero and Tejedor, 1995), we found *Sh* Kch to be expressed throughout development. The following two waves of expression were detected by WB: a small larval peak and a long second wave that began at the late pupal stage and reached a plateau in the adult. Accordingly, our immunohistological study starts with the third instar larval CNS. As shown in Figure 1*A*, the α -ShHX1 antiserum labels the neuropile over the entire whole-mount larval brain. The absence of labeling in the nervous system of *Df* flies (Fig. 1*B*) demonstrates the high specificity of this antiserum. The staining is particularly intense in the mushroom bodies (MB), which can be recognized easily by their characteristic morphology (Fig. 1*A,C,D*). Besides this wide and intense neuropile labeling, a small number of immunostained somata were detected at different focal planes throughout the

larval brain. This includes a well-defined distribution pattern of neurons (Fig. 1*D*, *blue spots*) such as those located in a medial plane close to the MB (Fig. 1*A,C*) as well as a few neuroblasts (Fig. 1*D*, *red spots*) that appear in thoracic and abdominal proliferative regions with a nonrepetitive pattern. Unfortunately, we have been unable to identify these cells, because their distribution pattern does not fit well with any map of neuronal families expressing particular neurochemical properties such as neurotransmitters and neuropeptides, or a given molecular marker (Buchner et al., 1986; White et al., 1986; Budnik and White, 1988; Vallés and White, 1988; Lundell and Hirsh, 1994).

To study the localization of the channels in more detail, we made serial semithin sections of immunostained larval brains and counterstained the somata with toluidine blue. This approach showed that the immunostaining is neither regular nor general throughout the neuropile (Fig. 1*E,F*). There are some areas clearly lacking in stain, such as the majority of transversal fibers that connect both hemispheres (Fig. 1*E*). It can also be observed that only a small number of cell bodies are actually immunostained (Fig. 1*E,F*). The labeling of cell bodies, particularly neuroblasts, was frequently found in intracellular locations (data not shown).

We have demonstrated previously by quantitative immunoblot assays that ~90% of *Sh* Kch in the adult fly are expressed in the head (Rogero and Tejedor, 1995). Therefore, we have approached the localization of these channels by serial sectioning and immunostaining of adult heads. In some cases, to improve the localization of the signal and its assignment to a given structure, immunolabeled sections were counterstained with toluidine blue.

The α -ShHX1 antiserum labels the retina, optic lobes (OL), and central brain throughout the whole head of the adult fly (Fig. 2*A–E*). The specificity of the immunolabeling again is demonstrated by the lack of any signal in the sections of *Df* flies (Fig. 2*F*). *Sh* Kch are observed in the following three general locations of the adult brain: neuropile, nerve fibers, and neuronal somata (Fig. 3). Examples of the first location include the wide distribution of immunostaining within the neuropile of the OL and central brain. However, the immunostaining is neither general nor homogeneous within each neuropile. As shown in Figures 2*C–E* and 3*B*, the pattern of immunostaining of the OL reveals the typical stratified tangential layers and the columnar organization of neuromeres that have been described extensively in *Drosophila* and other diptera (Ramon y Cajal and Sanchez, 1915; Strausfeld, 1976; Fischbach and Dittrich, 1989). Except the lamina, which lacks any signal, all OL neuromeres are labeled with varying intensity. At the medulla, layers 2 and 9 are highly labeled, whereas layers 4 and 6 show a lower intensity signal. The most distal layers of the lobula, all four layers of the lobula plate, and a fiber bundle located between the two, are also very strongly stained. In the central brain, the α , β , and γ lobes of the MB exhibit the strongest labeling of neuropile regions (Fig. 2*A*), whereas the calices and peduncles stain weakly (data not shown). The entire neuropile of the central complex is also well-stained, whereas labeling of the antennal lobe is almost completely absent (Fig. 2*B,J*). The central brain structure, which shows the strongest staining, is a series of axonal tracks (Figs. 3*A*, Fig. 2*C*) that run transversely, connecting both sides of the ventrolateral protocerebrum (Strausfeld, 1976). Similarly, most main fiber tracks connecting different regions of the nervous system such as the anterior optic nerves, which connect the OL with the protocerebrum (Fig. 2*D,G*), the cervical fiber linking brain and thoracic ganglia (Fig. 2*H*), and the posterior optic track, which connects both OL

Figure 1. Immunolocalization of *Sh Kch* in third instar larval brains. *A*, Immunostained brain from mature third instar *CS* larvae. *Small arrows* point to labeled somata. *B*, Parallel control of a *Df* brain. Photographs were taken at 200 $\times$ magnification from a ventral view. *C*, Higher magnification (400 $\times$) of the boxed area in *A*, showing immunolabeling of MB neuropile and a few cell bodies (*arrows*) at a medial focal plane. *D*, Schematic representation of the ventral–dorsal projection of a larval brain immunostained with anti-*Sh* serum. This contains the schematic localization of the most frequently immunolabeled cell bodies that was obtained with camera lucida drawing by moving throughout all focal planes of eight whole-mount brains. *Open circles* show the position of labeled cell bodies facing the ventral side of the brain, and *solid circles* represent cells occupying medial or dorsal positions. *Red and blue spots* correspond to neuroblasts and neurons, respectively. *Black areas* mark the location of MB neuropile, whereas *shaded regions* indicate total neuropile. *Arrows* mark the approximate positions of the semithin sections of *E* and *G*. *E*, Frontal section (4 μ m) at medial level of the OL and central brain from a larval brain, immunostained, dehydrated, and embedded as described in Materials and Methods. Semithin sections were cut and counterstained with toluidine blue. Ventral is to the top; *stars* mark unstained axonal fibers. *Arrow* points to a labeled cell body. Photograph was taken at 400 $\times$ magnification. *F*, Frontal section carried as in *E* at the level of thoracic segments. *GC*, Undifferentiated ganglion cells; *MB*, mushroom bodies; *N*, neuropile; *OPC*, outer proliferation center of the OL.

(data not shown), were strongly stained. However, a striking exception is the first optic chiasm, which links lamina and medulla and, as shown in Figure 3*B* (*star*), lacks any immunostain. Leg nerves, generated by the axons of thoracic motoneurons that

innervate leg muscles, are also extensively immunostained (Fig. 2*I*). We have also observed that most primary sensory afferents, including labro-frontal, ocellar nerves (data not shown), maxillo-labellar (Fig. 2*E*), antennal (Fig. 2*A,J*), and retinal inputs (see

Figure 2. Immunolocalization of *Sh Kch* in the nervous system of adult flies. Serial frontal cryostat sections of 48-hr-old adult heads were immunostained as indicated in Materials and Methods. *A–E*, Every third or fourth immunostained frontal section taken from a series throughout a head of a *w* fly showing labeling of antennal nerves (*AN*); anterior optic nerves (*AON*); central complex neuropile (*CC*); lobula neuropile (*LO*); maxillo-labellar nerves (*MLN*); medulla neuropile (*ME*); α , β , and γ lobes (*a*, *b*, and *g*, respectively) of the MB neuropile; retina (*RE*); and transversal fibers (*TF*). Lack of immunostaining of antennal lobula (*AL*) and lamina neuropile (*LA*) can be observed in *B* and *E*, respectively. *F*, Frontal section of an adult *Df* head at a level equivalent to that in *C*. Because this section completely lacks immunolabeling, the photograph was taken under Nomarski optics to make the tissue structure visible. The stained layer located between retina and lamina is the eye pigment of the basal lamina. *G*, High magnification showing anterior optic nerves connecting lobula (*LO*) and central brain (*CB*). *H*, Sagittal section at a medial plane through the head and thorax showing the brain including subesophageal ganglion (*SEG*), cervical fiber (*CF*), and thoracic muscles (*TM*). *I*, Sagittal section of a *CS* fly at the level of thoracic ganglia (*TG*) showing descending axons running along the leg nerve (*LN*). *J*, Sagittal section of a *CS* head at the level of the antenna (*A*). Beside the brain, other observable features are the strong labeling of antennal nerve (*AN*) and deutocerebrum (*DC*). Notice that the antennal lobe (*AL*) is practically deprived of staining.

below), are also immunostained by the anti-*Sh* antiserum. Strong staining of thick fibers running all along the antennal nerve can be observed in a sagittal section of the head (Fig. 2*J*). Most, if not all, of these labeled antennal fibers bypass the antennal lobula, the neuropile of which lacks immunostaining, before reaching the brain. The antennal nerve is formed by two bundles of mechanosensory and chemosensory fibers that move together from the antenna until they reach the brain, where they split into two tracks. One bundle is the olfactory track, which terminates at the antennal lobula; the other is the antennal mechanosensory and motor center track, which bypasses the antennal lobula terminating at the mechanosensory region of the deutocerebrum (Stocker RF et al., 1990). Accordingly, we have identified these labeled fibers as part of the antennal mechanosensory and motor center track.

In addition to the strong binding of anti-*Sh* antiserum to both neuropile and fiber tracks, we have also detected a third type of staining in the adult brain. This consists of staining of a small number of cell bodies that are spread throughout the cell body

rind of the central brain (Fig. 3*C*) and OL (data not shown) with no reproducible pattern of distribution.

The expression of *Sh Kch* at the retina deserves particular attention. Each ommatidium of *Drosophila* is a precise assembly of 8 photoreceptors and 11 accessory cells in a honeycomb-like matrix (for review, see Wolf and Ready, 1993). A horizontal section of retina immunostained with α -ShHX1 shows a radial pattern of intense labeling (Fig. 4*A*). Because the staining is observed below the layer of the lens and pseudocones and extends beyond the basal membrane to the lamina nuclear layer, labeling of the cone and pigment cells is very unlikely. However, the staining of mechanosensory bristles that project from alternate facet vertices cannot be ruled out. To test this, we have used the mutant *sc10.1*, which lacks all external mechanosensory organs of the head including all retinal sensilla (García-Bellido et al., 1979; Campuzano et al., 1985). As shown in Figure 4*B*, the staining pattern of retina remains in *sc* flies. Therefore, this is most consistent with labeling of photoreceptor neurons. Despite their similarities, the eight photoreceptor cells from *Drosophila omma-*

Figure 3. Different subcellular localization of *Sh* Kch in adult brain; counterstained sections. Cryostat sections of adult flies were immunostained with antiserum α -ShHX1 and counterstained with toluidine blue as described in Materials and Methods. *A*, High magnification of a counterstained section equivalent to that shown in Figure 2*C* showing the central brain at the level of transversal fibers. *B*, OL at 630 $\times$ magnification in a horizontal section at a medial level in an adult *w* head. An arrowhead marks the position of third optic chiasm. Small arrows point to immunostained photoreceptor axons crossing the lamina nuclear layer. A large arrow points to a labeled axonal bundle located between lobula and lobula plate. Numbers in the medulla neuropile correspond to tangential layers described by Fischbach and Dittrich (1989) that are immunolabeled. Notice that although the first optic chiasm (marked with a star) lacks any DAB stain, it can be clearly detected because of the typical $\times$ shape that the nerve bundles produce by passing between toluidine blue counterstained cell bodies of the medulla cortex. *C*, Detail at 1000 $\times$ magnification of a frontal section at the level of the fused body of the central complex, showing labeled cell bodies (arrows). FB, Fused body; La, lamina; Lo, lobula; Lp, lobula plate; Me, medulla; Oes, esophagus.

tidia can be classified into four classes (R1,3,4,6; R2,5; R7; and R8) (Wolf and Ready, 1993). A tangential section of the retina shows that α -ShHX1 stains only the complete inner periphery of each ommatidium (Fig. 4*C*). Because the ommatidia structure is fully preserved under the fixation conditions used (Fig. 4*E*), it can be concluded that *Sh* Kch expressed in retina are specifically located at the outer membrane of all eight photoreceptor neurons.

The ommatidial structure of the insect eye generates a retino-topic projection in the OL, which is constructed in a columnar way. R1–R6 photoreceptors project to the lamina neuropile, whereas R7 and R8 axons cross the lamina through the first optic chiasm and synapse at the medulla neuropile (Strausfeld, 1976; Fischbach and Dittrich, 1989). Although the axons of photoreceptors are labeled when they leave the retina and cross the lamina nuclear layer, avoiding the neuronal cell bodies, the staining disappears as soon as they enter the lamina neuropile (Figs. 3*B*, 4*A*). On the other hand, the medulla exhibits strong immunostaining (Fig. 3*B*). This raises the question of whether R7 and R8 photoreceptors express *Sh* Kch at their synaptic terminals. To address this, we have immunostained sections of *sevenless* mutant flies, which lack R7 photoreceptors (Tomlinson and Ready, 1986), and have found that the intense immunolabeling of defined medulla layers in the *wt* OL (Fig. 3*B*) is not modified in the mutant (Fig. 4*F*). These results suggest the almost exclusive location of *Sh* Kch in the cell body and axonal membranes of R1–R7 photoreceptors, and a low occurrence, or absence, at their synaptic terminals in lamina and medulla.

Are *Sh* Kch splice variants expressed selectively in different brain regions?

Because the antiserum α -ShHX1 recognizes *Sh* isoforms equally (Rogero and Tejedor, 1995), genetic tools were applied to answer the question of whether different *Sh* Kch variants produced at the *Sh* locus by alternative splicing are expressed differentially at a cellular level. Three well-studied *Sh* mutants, *T(X,Y)W32*, *T(X,Y)B55*, and *Sh*^{E62}, which modify the repertoire of *Sh* splice isoforms, have been used to test whether *Sh* Kch expression is abolished in specific brain structures or group of cells. As shown in Figure 5*A*, *W32* is a chromosomal translocation with the break-point located in the *Sh* transcription unit between exons 2 and 3 (Pongs et al., 1988; Schwarz et al., 1988). The *W32* translocation does not preclude the expression of all *Sh* Kch (Hardie et al., 1991). We have shown in a previous study by using high-resolution bidimensional electrophoresis and immunoblot techniques that at least two major *Sh* Kch proteins expressed in the adult *Drosophila* brain were eliminated by this mutation (Rogero and Tejedor, 1995). The immunohistological analysis of *W32* flies shows a general decrease in the intensity of CNS and retinal staining. This agrees with previous PCR experiments that showed that *Sh*B was the most abundant *Sh* variant in the adult head (Hardie et al., 1991). Distinct brain areas of *W32* flies, like most main fiber tracks (cervical fiber, anterior and posterior optic nerves, antennal nerves, and so forth), appear to show the same pattern of distribution as in *wt*. For example, the transversal fibers of the central brain can be compared in Figures 2*C* and 5*B*. However, clear differences between the *W32* and *wt* patterns are also found. For example, it is remarkable that the strong labeling of the MB neuropile of *wt* brains (Fig. 2*A*) is almost completely eliminated in *W32* (Fig. 5*C*). It must be emphasized that no ectopic distribution of immunolabeling was detected in any area of *W32* CNS. This strongly suggests that the translocation *W32* does not produce

Figure 4. Expression and localization of *Sh Kch* at the retina. High-magnification views (630 $\times$) of immunostained horizontal cryostat sections of adult *w* (*A*) and *sc* (*B*) heads. *Small arrows* in *A* point to immunostained photoreceptor axons crossing the lamina nuclear layer. These cannot be observed in *B* because of the eye pigment of the basal lamina (*arrowhead*) of *sc* flies. *C*, Immunostained tangential cryostat section of a *wt* retina. *D*, Schematic representation of the cellular components of an ommatidium. *E*, Plastic tangential semithin section of a *CS* retina, prepared as described in Materials and Methods, showing the conservation of ommatidial structure. *F*, OL of an immunostained horizontal section of a *sevenless* head at a level equivalent to that in Figure 3*B* but without counterstaining. *Br*, Bristle; *C*, pseudocone; *L*, corneal lens; *LA*, lamina; *ME*, medulla; *PC*, pigment cell; *R1–R7*, photoreceptor neurons.

secondary alterations in the pattern of *Sh Kch* expression. The chromosomal translocation *B55*, because of its location between the two groups of 3' alternative exons (Pongs et al., 1988; Schwarz et al., 1988), in principle should eliminate the C-terminal type 1 isoforms without affecting type 2 isoforms (see Fig. 5*A*). However, *B55* flies exhibited considerable ectopic expression of *Sh Kch* (data not shown), and therefore, this mutant was not used in this study. A good alternative to *B55* is the mutant *Sh^{E62}*, which has a point mutation in the acceptor site of one of the 3' alternative splice variants (Lichtinghagen et al., 1990) and, accordingly, modifies only the type 1 C-terminal isoforms (see Fig. 5*A*). Although the expression of *Sh Kch* in many areas of the *Sh^{E62}* nervous system, such as MB, central complex, retina, and so forth, does not seem to be modified by this mutation (data not shown), a few distinct features in the *Sh Kch* distribution can be found. The pattern of labeling of OL undergoes distinct changes in this mutant (Fig. 5*D*). For example, the staining of layers and columns seen in the *wt* medulla and lobula plate (Fig. 3*B*) is severely reduced and altered in *Sh^{E62}* (Fig. 5*D*). A more evident change is the almost complete elimination of labeling at the antennal nerve (compare Figs. 2*J* and 5*E*), whereas primary sensory afferents and connective fibers (maxillo-labelar, optic nerves, leg nerves, and so forth) were not modified (data not shown). Together, these results indicate that differences in the distribution of *Sh Kch* between *wt* and *W32* or *Sh^{E62}* brains are attributable to the lack of expression in the mutant flies of *Sh* isoforms, which seem to be preponderantly expressed in a few defined areas.

Late developmental expression of *Sh Kch*

We have shown in a previous work (Rogero and Tejedor, 1995) that the repertoire of *Sh Kch* splice variants expressed in embryo, larvae, and adult fly were rather different. Here, we have focused our attention on the second developmental wave of *Sh Kch* expression, because it is during this developmental period, from pupation to adult imago, that differentiation and definitive formation of the CNS structure occur (Kankel et al., 1980; Truman et al., 1993). We have analyzed, by immunoblot techniques, whether different *Sh* isoforms are expressed during metamorphosis and the beginning of imago life. As shown in Figure 6, changes in the pattern of detected proteins occur throughout this period, with a gradual increase in the relative intensity of larger MW bands, which are most abundant 48 hr after eclosion. This pattern remains stable at least for the next 72 hr (data not shown). Because we have shown previously that our procedures and control experiments preclude degradation of *Sh* proteins and that all protein bands detected by the antiserum α -ShHX1 are glycosylated to a similar extent (Rogero and Tejedor, 1995), it is unlikely that the complex *Sh* protein pattern shown in Figure 6 could be attributable either to proteolysis or to the maturation of a single protein caused by progressive glycosylation. To demonstrate that these changes in the electrophoretic pattern are a direct consequence of differential expression of *Sh Kch* splice variants, we have once again taken advantage of the mutant *W32*. As demonstrated previously in adult flies (Rogero and Tejedor, 1995), *W32*

Figure 5. Changes in the distribution of *Sh* Kch in mutants with modifications in the repertoire of *Sh* splice variants. *A*, Schematic representation of the *Sh* transcription unit with the approximate location of *B55*, *Sh^{E62}*, and *W32* mutations. *B*, Immunostained frontal cryostat section of an adult *W32* head at a level equivalent to that of Figure 2*C*. *C*, Immunostained frontal cryostat section of a *W32* head at the level of MB, equivalent to that in Figure 2*A*. *D*, Left hemisphere of an *Sh^{E62}* adult head as seen from an immunostained horizontal section. Notice the marked decrease in the immunostaining of OL neuromeres relative to the strong retinal labeling when compared with an equivalent *wt* section (Fig. 3*B*). The arrow points to fibers crossing between the lobula and lobula plate, that appear to have a level of *Sh* Kch expression similar to that found in *wt* flies. Numbers in the medulla neuropile are as in Figure 3*B*. Notice that in contrast to the *wt* medulla, layer 4 has the strongest labeling, whereas layers 2 and 9 are very weakly stained. *E*, Immunostained sagittal section through the head of an *Sh^{E62}* adult fly taken at a level equivalent to that in Figure 2*J*. *A*, Antenna; *AL*, antennal lobe; *AN*, antennal nerves; *LA*, lamina; *LO*, lobula; *LP*, lobula plate; *ME*, medulla; *MB*, mushroom bodies; *RE*, retina; *TF*, transversal fibers.

mutants also eliminate a subset of the *Sh* isoforms expressed in *CS* flies late in development (Fig. 6, arrows). Interestingly, this subset comprises at least one *Sh* isoform, the expression for which is developmentally regulated (Fig. 6). Together, these experiments strongly suggest that changes in the developmental *Sh* protein pattern are primarily attributable to the differential usage of some of the *Sh* Kch splice variants eliminated by *W32* (Fig. 5*A*, *ShB*, *ShD*). This also demonstrates that major changes in *Sh* band pattern actually occur after eclosion of the flies and, therefore, cannot be a consequence of neuronal development, because the CNS is considered to be fully formed and cells differentiated by this time (Kankel et al., 1980; Truman et al., 1993). Moreover, clear changes are detected when the fly is fully mature (8–48 hr). The question arises as to whether these band changes are the

result of gradual substitution of isoforms expressed in a given structure or cell group during differentiation or of the *de novo* expression of *Sh* Kch that is required by a cell group during a given developmental period, or even a combination of both mechanisms. To answer this question, we have focused on structures such as retina, antennal nerve, leg motoneuronal fibers, and MB, which show strong expression of *Sh* Kch in the adult fly and are functionally related to biological activities that are beginning, or significantly increasing, after eclosion.

The expression of retinal *Sh* Kch occurs after the appearance of the visual pigment. The retina of P12 pupa (bright red eyes) does not show α -ShHX1 labeling (Fig. 7*A*). At the P14–P15 stage, a few hours before eclosion, labeling could be detected for the first time (Fig. 7*B*). In recently eclosed flies, immunostaining was

Figure 6. Changes in the expression of *Sh* Kch isoforms during late development. Immunoblots of developmentally staged samples of CS and W32 flies from monodimensional SDS-PAGE gels. Experimental conditions were as published previously (Rogero and Tejedor, 1995). As indicated by the position of molecular weight standards, photographs show the region where, as demonstrated previously (Rogero and Tejedor, 1995), most *Sh* Kch proteins appear. LP, Late pupae; RE, recently eclosed fly; A7 and A48, adult flies 7 and 48 hr after eclosion. Arrowheads point to the position of an equivalent intense band in each blot, whereas arrows indicate the position of bands that disappear in W32 mutant flies.

intense (Fig. 7C), and during the first few hours of imaginal life, increased to the level found in the 48-hr-old retina. In contrast to the expression in retina, *Sh* Kch are detected from very early pupal stages at the OL neuropile (Fig. 7A) showing a pattern similar to that found in the adult brain (Fig. 2E). Thus, the expression of retinal *Sh* Kch appears to correlate with, or even slightly precede, the onset of sensory activity. Retina of flies, the late metamorphosis and manipulation of which were performed under dark conditions, shows a strong labeling (Fig. 7D), therefore excluding the possibility that light may trigger the expression of *Sh* Kch in photoreceptor cells.

At 18–20 hr after pupae formation, axons from differentiating antennal sensory neurons fasciculate and form the antennal nerve, which projects into the brain to complete the development of sensilla (Ray and Rodrigues, 1995). The expression of *Sh* Kch at the antennal nerve is first observed at the late pupal stage (P14–P15) as a weak staining (Fig. 7E) that increases in intensity after the eclosion of flies (Fig. 7F), reaching a plateau 24 hr later (data not shown). In contrast to changes in antennal nerves, general immunostaining of the brain does not vary much during this period (Figs. 7E,F, 2J).

Staining of axonal fibers from leg motoneurons is very weak at the late pupal stage (Fig. 7G), but becomes intense immediately after eclosion of the fly (data not shown), increasing to a maximum in the first few hours of imago life (Fig. 7H).

In contrast to retina, antennal nerves, and leg nerves, *Sh* Kch expression at the MB is very high as early as P9 pupae (Fig. 7I) and is maintained throughout development (data not shown).

DISCUSSION

Diverse functional roles can be inferred from the distribution and localization of *Sh* Kch

This study shows a major localization of *Sh* Kch in defined synaptic regions and axons. This is consistent with the electrophysiological phenotype of *Sh* mutants that produces modifications in neurotransmitter release (Jan et al., 1977) and action potentials (Tanouye et al., 1981). The most intense expression of

Sh Kch found in defined regions of the adult head such as MB and OL neuropile agrees with that found in an earlier immunohistochemical study (Schwarz et al., 1990). Although this previous work lacked analysis of many brain areas, there are some obvious differences with our results, such as the labeling of antennal lobe and lamina neuropiles and the lack of labeling of cell bodies and some axonal tracks reported by us. It is also striking that in the earlier study, antisera labeled a few nonspecific bands, but only a single specific band was detected in immunoblots of total *Drosophila* head proteins (Schwarz et al., 1990) in spite of the wide variety of different *Sh* splice variants generated at the *Sh* locus (Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988). Also, in the previous study, antisera could not detect *Sh* Kch in muscle cells, whereas our antiserum clearly labels postsynaptic *Sh* Kch at neuromuscular junctions (Tejedor et al., 1997). Thus, affinity and specificity deficits most probably can account for these contrasting results.

The preponderance of cellular localization of *Sh* Kch changes during development. In larval brains, *Sh* Kch are expressed at high levels throughout many synaptic areas and at low levels in some axonal fibers. By contrast, *Sh* Kch are abundant in most axonal fibers of the adult brain, whereas only a few synaptic regions appear to have these channels. This nonuniform localization in synaptic areas is supported further by comparison with the distribution patterns of synaptic proteins such as syntaxin (Cerezo et al., 1995), synaptotagmin, and synaptobrevin (DiAntonio et al., 1993). Together, this demonstrates a restricted expression of *Sh* Kch to certain neuronal populations, as suggested previously from electrical recordings performed in primary cultures (Baker and Salkoff, 1990) and studies performed with *Sh* transformant flies (Zhao et al., 1995).

Of particular interest is the high prevalence of *Sh* Kch in synaptic areas of lobula plate, MB, and central complex, structures implicated in integrative functions (Buchner et al., 1984; Heisenberg et al., 1985; Hanesch et al., 1989; Davis, 1993; Strauss and Heisenberg, 1993; Tully et al., 1994). The MB, for example, has been consistently related to associative olfactory learning (De Belle and Heisenberg, 1994). Because *Sh* Kch were not detected in the chemoreceptor axon fibers of the antennal nerve or in their synaptic connections at the antennal lobe, the altered olfactory behavior found in some *Sh* mutant alleles (Cowan and Siegel, 1986) must be attributable to the modified integration of sensory signals at the MB, rather than to abnormal transmission of the primary sensory signal. In the optic ganglia, *Sh* Kch are localized in a well-defined pattern. Schwarz et al. (1990) concluded that a single group of neurons (Y cells) could account for much of the *Sh* Kch pattern of expression in this tissue. However, this cannot be the case, because Y cells have been described as having no arborization endings at the distal medulla (Fischbach and Dittrich, 1989), where we have found two prominently labeled synaptic layers (layers 2 and 4). In contrast, our results favor the restriction of *Sh* Kch expression to a given retinotopic projection. Among the four different visual pathways that have been found in *Drosophila* (Bausenwein et al., 1992), most of the *Sh* Kch distribution at the OL fits very nicely with projections of pathway 2 neurons. This possible restricted expression may have interesting functional implications, because it has been suggested that different retinotopic projections are involved in the computation of color, shape, motion, and so forth (Buchner et al., 1984; Bausenwein et al., 1989).

The functional role of those *Sh* Kch found in neuronal cell bodies remains uncertain. Because we have frequently found them

Figure 7. Changes of *Sh* Kch distribution during late development. *A*, Immunostained frontal section showing the right hemisphere of a CS P12 pupae at a level close to that shown in Figure 2*E* for an adult head. *B*, Idem for a P14–P15 pupae. *C*, Id for a recently eclosed fly. *D*, CS late third instar larvae were collected and pupation allowed to progress under normal conditions until P5. Development from P5 until ~48 hr after eclosion was performed in the dark. Flies were then collected and rapidly embedded (1–2 min) for immunohistology under red light, to which *Drosophila* photoreceptors are insensitive (Menne and Spatz, 1977). *E*, Sagittal section through the head of a CS P15 pupa at the level of the antennal nerve. This section is equivalent to that of Figure 2*J*. *F*, Id for a 7-hr-old imago. Notice the change in the relative intensities of brain and antennal nerve labeling when compared with *E*. *G*, Sagittal section of a CS P15 pupa at the level of the thoracic ganglia and leg nerves. *H*, Id for a recently eclosed fly. *I*, Immunostained frontal section of a CS P9 pupa at the level of MB. Abbreviations are as in Figure 2.

in intracellular locations, a transient expression or an intracellular pool of *Sh* Kch could be likely explanations. The rapid induction of Kch after certain stimuli and the short half-life of some Kch mRNAs (Takimoto et al., 1993) make this feasible. Interestingly, the relationship of voltage-sensitive Kch to the cell cycle has been reported extensively (for review, see Dubois and Rouzair-Dubois, 1993). Thus, a transient expression of *Sh* Kch related to the cell cycle of neuroblasts is an attractive hypothesis.

Electrical recordings in *Drosophila* photoreceptors have found a *Shaker* current and two noninactivating K^+ currents (Hardie, 1991). We have shown here that *Sh* Kch are located in the outer

membrane and are absent from lumen and rhabdomere membranes. Because it has been suggested that the two non-*Sh* Kch are located in the internal lumen membranes (Hardie, 1991), *Drosophila* photoreceptors may provide a good model system to study the mechanisms involved in the cellular segregation of ion channels.

***Sh* Kch splice variants are expressed differentially but not completely segregated**

The formation of heteromultimeric Kch by the assembly of different subunits has been proposed as a mechanism for the gen-

eration of Kch diversity (Isacoff et al., 1990; Ruppersberg et al., 1990). However, there is compelling evidence in the literature to support both the colocalization and the segregation of different Kch subunits in mammalian brain (Sheng et al., 1992, 1994; Wang et al., 1993, 1994; Scott et al., 1994; Mi et al., 1995; Weiser et al., 1995). PCR (Hardie et al., 1991), *in situ* hybridization (Tseng-Crank et al., 1991), and reporter gene engineering (Mottes and Iverson, 1995) experiments indicate that *Sh* splice variants can be expressed differentially in some areas of the nervous tissue, retina, and muscle of *Drosophila*. An immunohistological study on adult brain has shown that structures stained with an antiserum against a single *Sh* isoform were a subset of those labeled with a general anti-*Sh* antiserum (Schwarz et al., 1990), therefore suggesting a segregation of splice variants. However, it was not shown that the protein bands detected by WB with the isoform-specific serum were also a subset of the bands labeled by the general antiserum. Our experiments with mutants that selectively eliminate a few of the alternative *Sh* splice isoforms have shown that only in antennal nerve axons of the mutant *ShE62* is the expression of *Sh* Kch apparently abolished. More typically, the expression is reduced by varying degrees in most brain regions of *ShE62* and *W32* flies. Therefore, it can be concluded that there is a wide differential usage of the repertoire of *Sh* splice variants, but that complete segregation takes place only in a few brain regions.

Differential spatio-temporal expression of *Sh* Kch isoforms and their role during late development

Large modifications of neuronal electrical properties during development have been recorded in various animals. Voltage-gated currents appear in a sequence characteristic of each neuronal population (for review, see Spitzer, 1991; Spitzer et al., 1994). Kch have been detected as early as neural induction (Ribera, 1990) and during development undergo abrupt changes in density and kinetics (Barish, 1986; Harris et al., 1988; Yool et al., 1988).

There are no reports in the literature on the differentiation of electrical properties of *Drosophila* CNS neurons, although some data are available from photoreceptor neurons (Hardie, 1991) and pupal muscle (Salkoff and Wyman, 1981). Previous work (Rogero and Tejedor, 1995) and results presented here clearly show that *Sh* Kch are expressed differentially throughout development of the *Drosophila* nervous system. In contrast to myotube differentiation, in which two *Sh* conductances with different electrical properties were recorded showing a progressive replacement of an early type I_A by a late mature one (Broadie and Bate, 1993), we have shown that major changes in neuronal expression of *Sh* Kch during late development are not attributable to the replacement of *Sh* isoforms. Our results suggest a *de novo* expression of *Sh* isoforms that seems to correlate with the increase in sensory and motor activity of newborn flies. Thus, the expression of *Sh* Kch may be one of the last refinements in the differentiation of neuronal electrical properties and, in addition, may be involved in adaptative mechanisms of excitability, as suggested by the wide changes in the expression of *Sh* isoforms in adult flies.

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