The LIM-Homeodomain Protein Islet Dictates Motor Neuron Electrical Properties by Regulating K⁺ Channel Expression

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http://dx.doi.org/10.1016/j.neuron.2012.06.015
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SUMMARY

Neuron electrical properties are critical to function and generally subtype specific, as are patterns of axonal and dendritic projections. Specification of motoneuron morphology and axon pathfinding has been studied extensively, implicating the combinatorial action of Lim-homeodomain transcription factors. However, the specification of electrical properties is not understood. Here, we address the key issues of whether the same transcription factors that specify morphology also determine subtype specific electrical properties. We show that Drosophila motoneuron subtypes express different K⁺ currents and that these are regulated by the conserved Lim-homeodomain transcription factor Islet. Specifically, Islet is sufficient to repress a Shaker-mediated A-type K⁺ current, most likely due to a direct transcriptional effect. A reduction in Shaker increases the frequency of action potential firing. Our results demonstrate the deterministic role of Islet on the excitability patterns characteristic of motoneuron subtypes.

INTRODUCTION

Diversity in neuronal signaling is critical for emergence of appropriate behavior. This diversity is reflected in dendrite morphology, axon pathfinding, choice of synaptic partners, transmitter phenotype, and cocktail of ion channels expressed by individual neurons. Many aspects of vertebrate (e.g., chick, zebrafish, and mouse) motoneuron development, including cell specification, axonal pathfinding, and neurotransmitter choice are regulated through expression of LIM-homeodomain transcription factors, including Islet1/2, Lim1/3, and Hb9 (Appel et al., 1995; Hutchinson et al., 2007; Pfaff et al., 1996; Segawa et al., 2001; Song et al., 2009; Thaler et al., 2004). Homologous proteins, and additional homeodomain (HD) proteins such as Even-skipped (Eve), serve similar functions in invertebrate motoneurons (e.g., C. elegans and Drosophila) (Certel and Thor, 2004; Esmaeili et al., 2002; Fujioka et al., 2003; Landgraf and Thor, 2006; Odden et al., 2002; Thor and Thomas, 1997, 2002). However, the extent to which neuronal electrical properties are similarly predetermined as part of cell-intrinsic developmental mechanisms remains unknown.

Neurons grown in culture often express their normal complement of both voltage- and ligand-gated ion channels (O’Dowd et al., 1988; Ribera and Spitzer, 1990; Spitzer, 1994). This suggests a significant degree of cell autonomy in the determination of electrical properties that presumably facilitates initial network formation. Once part of a circuit, however, such neurons become exposed to synaptic activity. As a result, predetermined electrical properties are modified by a variety of well-described mechanisms (Davis and Bezprozvanny, 2001; Spitzer et al., 2002). Such tuning ensures consistency of network output in response to potentially destabilizing activity resulting from Hebbian-based synaptic plasticity (Turrigiano and Nelson, 2004). The formation of functional neural circuits would seem, therefore, critically reliant on both intrinsic predetermination and subsequent extrinsic activity-dependent mechanisms to shape neuronal electrical properties. Key to understanding how intrinsic and extrinsic mechanisms are integrated will be the identification of factors that regulate predetermination.

The fruitfly, Drosophila, has been central to studies that have identified intrinsic determinants of neuronal morphology. Within the Drosophila central nervous system (CNS) the transcription factor Islet is expressed in the RP1, RP3, RP4, and RP5 motoneurons (termed ventral motoneurons, vMNs) that project to ventral muscles (Broihier and Skeath, 2002; Landgraf and Thor, 2006; Thor et al., 1999). By contrast, motoneurons projecting to dorsal muscles (e.g., aCC and RP2, termed dorsal motoneurons, dMNs) express a different homeodomain transcription factor, Even-skipped (Eve) (Broihier and Skeath, 2002; Landgraf et al., 1999). Misregulation of these transcription factors is sufficient to alter subtype-specific axonal projections (Broihier and Skeath, 2002; Landgraf et al., 1999). Thus, Eve and Islet constitute what might be considered a bimodal switch with each being deterministic for either dorsal or ventral-projecting motor axon trajectories, respectively.

Here, we report that the presence of Islet is also deterministic for expression of Shaker (Sh)-mediated outward A-type K⁺ current. The vMN and dMN subgroups differ in magnitude of outward K⁺ currents recorded by whole-cell patch clamp. We show that this difference is maintained by endogenous expression of islet.
in the vMNs. We also show that Islet is sufficient to repress expression of a Sh-mediated K+ current. By contrast, dMNs, which do not express islet, exhibit a robust Sh-mediated K+ current. The deterministic function of Islet is evidenced first by the fact that loss of function results in a transformation of total outward K+ current in the vMNs to mirror that present in dMNs. Second, ectopic expression of islet in dMNs or body wall muscle is sufficient to repress expression of the endogenous Sh-mediated K+ current. Thus, in addition to being sufficient to predetermine aspects of neuronal connectivity, Islet is sufficient to specify electrical properties in those neurons in which it is expressed.

RESULTS

Dorsal and Ventral Motoneuron Subgroups Show Specific K+ Current Profiles

A crucial test of the hypothesis that Islet regulates ion channel gene expression is the demonstration that membrane electrical properties of Islet-expressing vMNs differ to those of Eve-expressing dMNs. To determine if this is true, we recorded total K+ currents from both motoneuron subtypes in first-instar larvae (1–4 hr after hatching; see Figure 1A). Motoneurons were initially identified on the basis of their medial dorsal position in the ventral nerve cord; following electrophysiological patch clamp recordings precise subtype was confirmed on the basis of axonal projection that was visualized by dye filling. We did not observe differences within either subgroups; therefore, recordings have been pooled for the vMN or dMN subtypes.

Figure 1B shows averaged total outward K+ currents recorded from both the dMNs and vMNs. The outward K+ current is composed of a fast-activating and inactivating component, (IKfast, indicated by the arrow in Figure 1B) and a slower-activating, non-inactivating component, (IKslow, indicated by the box in Figure 1B). Analyzing current densities for IKfast and IKslow (Figure 1C) shows that dMNs have significantly larger outward K+ currents compared to vMNs (Figure 1C; at holding potential of +40 mV IKfast: 60.1 ± 4.3 versus 42.6 ± 3.1 pA/pF; IKslow: 49.0 ± 4.4 versus 33.3 ± 2.4 pA/pF, dMNs versus vMNs, respectively, p < 0.01). Thus, vMNs and dMNs differ in their electrical properties.

The CNS of a first-instar larva is a mature functional neural network in which synaptic transmission is active. Hence, the
differences we observe in K+ currents could be established entirely due to network activity. Alternatively, subtype specificity might be determined prior to neuronal network formation and, as such, could be considered an intrinsic property of the specific motoneurons. To determine this experimentally, we repeated our analysis following complete block of synaptic transmission (i.e., absence of network activity), achieved through expressing tetanus toxin light chain (TeTxLC) throughout the entire CNS. Using the GAL41407 driver, TeTxLC was expressed pan-neurally starting at the early neuroblast stage. Since TeTxLC-expressing embryos do not hatch, we recorded K+ currents just prior to expected hatching (at late embryonic stage 17). At this stage motoneurons have become fully functional components of the motor network (Baines and Bate, 1998). We found that IK was not significantly perturbed, in either dMNs or vMNs, by blockade of synaptic release. Moreover the difference in K+ currents between the dMNs and vMNs was maintained for both IKfast and IKslow. That differences in IK levels between dMNs and vMNs are established and maintained in the absence of synaptic release strongly suggests that they arise from intrinsic developmental mechanisms independent of evoked synaptic transmission.

Islet Determines the Electrical Properties of Ventrally Projecting Motoneurons

Drosophila larval motoneurons that project axons to ventral muscles express Islet, while those that innervate dorsal muscles express Eve. Loss of islet is sufficient to direct ventral-projecting axons dorsally and loss of eve to direct dorsal-projecting axons ventrally (Landgraf et al., 1999; Thor and Thomas, 1997). These two distinct motoneuron subtypes provide, therefore, a tractable system to test whether the differences we observe in K+ conductance is also intrinsically determined. In order to test whether Islet is able to influence K+ currents we recorded from vMNs in an islet null (islet /- ) mutant. This analysis indicated that Islet is sufficient to regulate K+ conductance in these motoneurons. Thus, peak current density for IKfast was significantly increased in homozygous islet /- mutants (Figure 2A; WT 42.6 ± 3.1 versus...

Figure 2. Islet Regulates K+ Currents in Ventral, but Not Dorsal, Motoneurons

(A) Shows composite averaged K+ currents (representing the average from at least eight individual neurons) and respective IV plots for WT and islet /- mutant vMNs. Voltage-clamp protocol as in Figure 1. Current density of IKfast of vMNs (obtained from a prepulse of −90 mV) is significantly larger in islet /- compared to WT at all test potentials above −40 mV. (B) Neurons were subjected to a prepulse of −20 mV to inactivate IKfast. The remaining IKslow of vMNs is indistinguishable between islet /- and WT. (C) Measurement of IKfast (obtained from a prepulse of −90 mV) in dMNs in islet /- and WT are not different. Values shown are means ± SEM (n ≥ 8). (D) Averaged responses of WT dMNs, WT vMNs, and islet /- vMNs evoked by the highest test potential (−90 mV prepulse and +40 mV test) are superimposed. The absence of islet from vMNs increases K+ current magnitude to WT dMNs levels. Scale bars are 20 pA/pF and 10 ms for voltage-clamp responses and 100 mV/10 ms for voltage-clamp protocol.
**Islet Regulates Electrical Properties**

Islet 62.6 ± 5.8 pA/pF (p ≤ 0.05). By contrast, I_{Kfast} in heterozygous siblings (+/−) did not differ from WT (data not shown). To better measure I_{Kslow}, we inactivated I_{Kfast} by applying a −20mV prepulse (100 ms; see Barnes and Bate, 1998). Figure 2B shows that loss of islet had no effect on I_{Kslow} (WT 24.2 ± 2.3 versus islet 28 ± 3.9 pA/pF (p = 0.45). We also compared voltage-gated inward currents (i.e., I_{Na} and I_{Ca}) in vMNs of heterozygous islet−/− and homozygous islet−/− mutants. Loss of islet did not affect the peak current densities of either current (I_{Na}: −23.4 ± 2.7 versus −19.7 ± 2.4 and I_{Ca}: −19.69 ± 1.68 versus −21.03 ± 2.43, islet−/− versus islet−/−). Thus, loss of islet results in a selective increase in only I_{Kfast} in the vMNs.

To test for autonomy of effect, we also recorded from dMNs in the islet−/− mutant. Dorsal MNs do not express islet, and I_{Kfast} currents of WT and mutant larvae were statistically indistinguishable (Figure 2C; WT 60.1 ± 4.3 pA/pF versus islet−/− 68.2 ± 5.9 pA/pF (p = 0.28). We conclude that loss of islet only affects I_{Kfast} in vMNs in which it is normally expressed, but not in dMNs that lack expression of this transcription factor. We further noted that loss of islet from the vMNs resulted in a transformation of I_{Kfast} to recapitulate the magnitude of this same current recorded in dMNs. When averaged responses of islet−/− vMNs and WT dMNs were superimposed, only small kinetic differences remain (Figure 2D). Such an observation is entirely consistent with, and indeed predictive of, the magnitude of I_{Kfast} being regulated by endogenous expression of Islet.

**Islet Represses a DTx-Sensitive Current**

Fast K+ currents in Drosophila neurons are encoded by one or more of at least three different genes: two voltage-gated fast-activating and inactivating channels (A-currents) termed Shal and Shaker (Sh) and a Ca2+-activated BK channel termed slowpoke (Baker and Salkoff, 1990; Elkins et al., 1986; Singh and Wu, 1990). To determine which K+ current is increased in activating and inactivating channels (A-currents) termed more of at least three different genes: two voltage-gated fast- and repressed by Islet. To do so we added Cd2+ to the bath solution.

Our data are consistent with Islet acting to repress expression of Sh in vMNs. Moreover, removal of this repression results in expression of Sh-mediated K+ channels that confer “dorsal-like” electrical properties. This model posits, therefore, that dMNs normally express a Sh-mediated K+ current.

To test this, we compared I_{Kfast} in dMNs between WT and in the presence of either DTx or in a Sh null mutant (Sh−/−). We performed these recordings in the presence of external Cd2+ to block Ca2+-activated fast K+ currents. Both acute block of Sh activity (DTx) and loss of function of Sh expression significantly reduced I_{Kfast} (Figure 3B; WT 40.5 ± 1.9 versus WT + DTx 29.3 ± 2.7 versus Sh−/− 26.1 ± 1.7 pA/pF; p ≤ 0.01 and p ≤ 0.01, respectively). Moreover, the I_{Kfast} recorded in dMNs under both conditions (WT + DTx 29.3 ± 2.7 and Sh−/− 26.1 ± 1.7 pA/pF) was indistinguishable from that of in vMNs in WT (26.1 ± 2.3 pA/pF, DTx p = 0.38, Sh = 1), which is in full agreement with our model. To further support the notion that the difference in I_{Kfast} that exists between dMNs and vMNs is due, at least in part, to expression of Sh in dMNs, we recorded I_{Kfast} in vMNs under the same conditions. As expected, neither the presence of DTx, nor loss of Sh, had any marked effect on I_{Kfast} in vMNs (p = 0.51 and 0.23, respectively; Figure 3B).

To further verify the differential expression of Sh in dMNs versus vMNs we assessed transcription of Sh in these two cell types by in situ hybridization. We designed probes that specifically recognize the Sh pre-mRNA. These intron probes label the unspliced Sh transcript at the site of transcription within the nucleus, but not the fully mature message in the cytoplasm. We detected Sh transcription in dMNs, labeled with Eve antibodies (Figure 3C, black arrowheads), but not in vMNs, labeled by expression of GFP (Lim3 > nlsGFP; Figure 3D, white arrowheads). Taken together, both electrophysiology and in situ hybridization are consistent with dMNs expressing Sh while the vMNs do not.

**Islet Is Both Necessary and Sufficient to Repress Sh-Mediated K+ Currents**

Next, we tested whether Islet is sufficient to repress Sh-mediated K+ currents in cells where Sh, but not islet, is normally expressed. We used two different preparations for these experiments. First, we ectopically expressed islet in dMNs. Driving a UAS-islet transgene with GAL4D02-α significantly reduced I_{Kfast} (34.4 ± 2.6 versus 41.2 ± 1.9 pA/pF, experimental versus controls which consisted of WT and heterozygous GAL4 driver line; p ≤ 0.05; Figure 4A). These recordings were carried out in the presence of external Cd2+ to eliminate Ca2+-dependent K+ channels.

The observed reduction in I_{Kfast} in dMNs could, however, be due to a reduction in either Sh- or Shal-mediated K+ currents. To distinguish between these two possibilities, we tested for DTx sensitivity, which is observed in WT dMNs and is an indicator for the presence of Sh currents. DTx sensitivity was lost when islet was ectopically expressed in dMNs (Figure 4A). These recordings were carried out in the presence of external Cd2+ to eliminate Ca2+-dependent K+ currents.

The second preparation we used takes advantage of the fact that I_{Kfast} in body wall muscle is solely due to Sh and Slowpoke (the latter of which can be easily blocked [Singh and
We recorded from muscle 6 in abdominal segments 3 and 4 in first-instar larvae. To remove the $I_{K_{\text{slowpoke}}}$ component and hence isolate the Sh-mediated $I_{K_{\text{fast}}}$, recordings were done in low calcium (0.1 mM) external saline. Figure 4B depicts the averaged responses from voltage-clamp recordings in control muscle (heterozygous GAL424B driver, upper trace) and muscle expressing islet (lower trace). Peak current densities of $I_{K_{\text{fast}}}$ (entirely due to Sh-mediated $K^+$ current) and the slow noninactivating currents recorded at +40 mV are shown in Figure 4C. Ectopic expression of islet in muscle is sufficient to produce a significant reduction in $I_{K_{\text{fast}}}$ (control 26.6 ± 2.4 versus 24B > islet 15.8 ± 1.0 pA/pF, $p \leq 0.01$) while no effect was seen on the slow current. Thus, expression of islet in dMNs is sufficient to reduce a DTx-sensitive component of $I_{K_{\text{fast}}}$. Similar expression in muscle clearly demonstrates that Islet is sufficient to downregulate a Sh-mediated fast $K^+$ current.

### Islet Binds Directly to the Sh Locus

Our electrophysiology indicates that Islet is able to repress Sh-mediated $K^+$ current. To identify putative targets of Islet we used DamID, a well-accepted technique for demonstrating direct binding to chromatin or DNA in vivo (Choksi et al., 2006; Filion et al., 2010; Southall and Brand, 2009; van Steensel and Henikoff, 2000). Our analysis identifies 1,769 genes (exhibiting...
one or more peaks of Islet binding within 5 kb of the transcriptional unit) as direct targets of Islet (FDR < 0.1%). Consistent with our model of Islet regulating a Sh-mediated K⁺ current, we find three significant binding sites within introns of the Sh locus (arrows 1 to 3 in Figure 5). Intragenic binding of transcription factors is common in both vertebrates (Robertson et al., 2007) and invertebrates (Southall and Brand, 2009). A fourth significant peak is found upstream of Sh (arrow 4 in Figure 5). Binding of Islet at this site could regulate the expression of either Sh and/or CG15373 an adjacent, divergently transcribed, gene. By contrast, Shal and slowpoke, which also encode fast neuronal K⁺ currents, were not identified as putative targets (Figure 5). Thus, these data show that Islet binds to the Sh locus and is likely to regulate transcription of the Sh gene directly.

To confirm that Islet binds Sh and regulates its transcription, we used qRT-PCR to quantify levels of Sh transcripts. We compared Sh transcript levels in larval CNS between control, islet⁻/⁻ and panneuronal islet expression (1407 > islet). In comparison to control, the absence of islet⁻/⁻ resulted in a 27% increase in Sh (1.27 ± 0.01, n = 2, p < 0.05). By contrast, panneuronal expression of transgenic islet resulted in a 45% decrease in Sh transcript (0.45 ± 0.06, n = 2, p < 0.05). We also measured Sh transcript level in body wall muscle following ectopic expression of islet (24B > islet). Similar to the CNS, Sh transcripts were reduced by 31% relative to control (0.31 ± 0.01, n = 2, p < 0.05). Taken together with the results obtained by DamID, this strongly suggests that Islet binds to, and represses transcription of, the Sh gene.

**Sh Regulates Action Potential Frequency**

Voltage-dependent K⁺ currents, such as those mediated by Sh, contribute to setting membrane excitability (and thus the ability to fire action potentials) (Goldberg et al., 2008; Peng and Wu, 2007). These currents are therefore critical for network function and the generation of appropriate behaviors (Smart et al., 1998). It has been shown that modulation of Sh-mediated current, using dominant-negative transgenes, can bring about significant changes in excitability (Mosca et al., 2006). We were interested in whether and how excitability differs between motorneurons that express a Sh-mediated K⁺ current (dMNs) and those that do not (vMNs). We recorded excitability in current clamp. Typical responses are shown in Figure 6A. We found that dMNs fired significantly fewer action potentials than vMNs at most current steps (Figure 6B; 10 pA: 18.2 ± 0.9 versus 22.1 ± 1.4 p = 0.04; 8 pA: 15.3 ± 1.0 versus 19.1 ± 1.1 p = 0.02; 6 pA: 11.5 ± 1.0 versus 15.2 ± 1.2 p = 0.04; 4 pA: 6.5 ± 1.2 versus 9.9 ± 1.4 p = 0.09; 2 pA: 0.8 ± 0.3 versus 3.8 ± 1.0 p = 0.03; 1 pA: 0.1 ± 0.1, versus 0.9 ± 0.4: p = 0.13; dorsal versus ventral, respectively). The above results suggest that the Sh-mediated K⁺ current (expressed only in dMNs) reduces action potential (APs) firing when present.

To validate this conclusion, we reduced Sh current in dMNs acutely by adding DTx to the bath and recorded AP firing. AP firing increased from 18.2 ± 0.9 APs (WT) to 25.7 ± 1.9 APs (DTx, p < 0.05; Figure 6C). A similar result, although not significant, was obtained when APs were recorded from dMNs in a Sh mutant (18.2 ± 0.9 to 21.2 ± 1.5 APs, p = 0.07; Figure 6C). Indeed, in both treatments, firing rates between dMNs and vMNs were indistinguishable (Sh⁻/⁻ 21.2 ± 1.5 versus 22.7 ± 1.1; DTx 25.7 ± 1.9 versus 23.0 ± 1.8 APs, dMNs versus vMNs respectively, p > 0.05). As predicted, vMN excitability was not affected by either DTx or loss of Sh (22.1 ± 1.4 versus 23.0 ± 1.8 versus 22.7 ± 1.1, WT, DTx, Sh⁻/⁻, respectively.)
p > 0.05; Figure 6C). Perhaps unexpectedly, the increase in \( I_{K_{fast}} \) in vMNs, which results from the loss of \textit{islet}, did not influence AP firing. Loss of \textit{islet} also had no effect on APs fired in dMNs which is predictable because dMNs do not express this protein (Figure 6C). Finally, determination of AP firing in a \textit{Sh;islet} double loss of function mutant revealed no additional effects: AP firing is increased in dMNs and unaffected in vMNs (data not shown). Why loss of \textit{islet}, which increases \( I_{K_{fast}} \) in vMNs, does not influence AP firing in these neurons is unknown, but may be indicative of additional homeostatic mechanisms.

**DISCUSSION**

Diversity in neuronal electrical properties is dictated by the type, location, and number of ion channels expressed in individual neurons. While activity-dependent mechanisms that act to adjust these properties in mature neurons have been studied in detail (Davis and Bezprozvanny, 2001; Spitzer et al., 2002), the mechanisms that specify electrical properties in embryonic neurons, prior to network formation, are not understood. These mechanisms are, however, likely to be part of cell-intrinsic programs of specification. The demonstration of differential expression of transcription factors between neuronal cell types underpins the proposal of a combinatorial code sufficient to determine key aspects of neuron specification, including axon guidance and neurotransmitter phenotype (Polleux et al., 2007; Shirasaki and Pfaff, 2002; Thor and Thomas, 2002). However, whether these same factors are sufficient to set cell-specific electrical characteristics remains unknown.

A wealth of studies on motoneuron specification, from flies to mammals, has shown that early developmental decisions, such as subclass identity, is dictated, at least in part, by a code of transcription factors (Dasen et al., 2005, 2008; De Marco Garcia and Jessell, 2008; Landgraf et al., 1999; Landgraf and Thor, 2006; Thor and Thomas, 1997). With its relatively simple CNS and powerful molecular genetics, \textit{Drosophila} has been central to these studies. Embryonic \textit{Drosophila} motoneurons express a stereotypic mix of identified transcription factors which are evolutionary conserved with mammals (Thaler et al., 1999, 2002; Thor and Thomas, 1997). Motoneurons which predominantly innervate ventral muscles express \textit{islet}, \textit{Lim3}, and \textit{dHb9}. Motoneurons which project dorsally express \textit{eve} (Landgraf et al., 1999; Landgraf and Thor, 2006; Thor and Thomas, 1997). A first indication that ion channel genes may also be targets of these transcription factors was provided by our demonstration that overexpression of \textit{eve} was sufficient to alter the outward voltage-gated \( K^+ \) current through transcriptional repression of \textit{slowpoke} (encoding a BK \( Ca^{2+} \)-activated \( K^+ \) channel) in \textit{Drosophila} motoneurons (Pym et al., 2006). However, while a common developmental regulation of neuronal morphology and function, at least in motoneurons, might be inferred from this study, only Eve-positive cells were investigated. This leaves open the question, whether Eve, or for that matter any of the other transcription factors, is deterministic for specific membrane currents.

The principle of duality in role for transcription factors such as Eve and Islet is significant because it is predictive that neuron morphology and electrical signaling are, at least in part, determined by common developmental mechanisms. Studies of vertebrate homologs of these transcription factors, widespread in the mammalian CNS, provide additional support for such a scenario. For example, Islet-1 and Islet-2 are known to regulate neuron identity, axonal guidance and choice of neurotransmitter...
in vertebrate CNS (Hutchinson and Eisen, 2006; Segawa et al., 2001; Thaler et al., 2004). Associated microarray analysis on murine mutant tissue identifies ion channels as putative targets of Islet-1, including Shal-related K⁺ channel Kcnzd2 and Na⁺ channel Na₄.1.8. Regulation of expression has, however, yet to be demonstrated (Sun et al., 2008). It is conceivable that in zebrafish recently reported differences in outward K⁺ currents between two embryonic motoneurons, dorsal MIP and ventral CaP (Moreno and Ribera, 2009), may be regulated by the differential expression of Islet1/2 in these neurons (Appel et al., 1995).

We provide substantial evidence that differential expression of islet in vMNs versus dMNs is critical for determining subtype-specific differences in Sh-mediated K⁺ currents. Because these Sh-mediated K⁺ currents regulate action potential frequency, they will contribute to network function. Comparable to our findings in Drosophila, in both the mouse cochlea and cortex, neurons that fire only a small number of action potentials to a given current pulse (termed rapidly adapting) express a DTx-sensitive Kv1 (Sh-like) K⁺ current. By contrast, neurons that fire many action potentials (slowly adapting) do not. The firing pattern of rapidly adapting neurons can be transformed into that of slowly adapting neurons by application of the Sh-specific blocker DTx (Miller et al., 2008). Our own data are consistent with such a role for Sh because we show that dMNs which express Sh, fire fewer action potentials than vMNs. Moreover, the number of action potentials fired by dMNs is increased by genetic or pharmacological block of the Sh-mediated K⁺ current. We envisage, therefore, that regulation of action potential firing, through Islet-mediated transcriptional control of a Sh-like K⁺ current, might be well conserved.

While the presence of early factors able to regulate ion-channel gene expression is predictive of predetermination of electrical signaling properties in embryonic neurons, a challenge remains to understand how individual neurons decode this information. In the Drosophila ventral nerve cord, we find that the presence or absence of a Sh-mediated K⁺ current is determined by whether islet is expressed or not. Thus, Islet seems to act as a binary switch; when present it prevents expression of Sh and vice versa. However, it seems unlikely that all combinatorial factors act in this way. For example, the activity of Eve seems to be related to its relative level of expression, since endogenous Eve only partially represses transcription of slowpoke (a Ca²⁺-dependent K⁺ channel) in the dorsal motoneuron aCC (Pym et al., 2006). It remains to be determined whether efficacy of regulatory activity is specific to individual transcription factors or to target genes.

We show here that the Lim-homeodomain transcription factor Islet forms part of an intrinsic “decision-making” process that is critical to specifying subtype-specific electrical properties in developing motoneurons. It might be argued that input from pre- and postsynaptic partners is involved in setting early electrophysiological differences between neurons. Indeed such inputs play a pivotal role during axonogenesis and synapse development. Blocking all synaptic transmission showed that neural network activity is not required to establish early electrophysiological differences between motoneuron subgroups. Motoneurons also receive instructive cues from their postsynaptic muscle targets during NMJ development (Fitzsimonds and Poo, 1998). In this regard it is significant that the difference in k_{fast} we observe between dMNs and vMNs is abolished in a myosin heavy chain mutant (mhc1) that fails to produce contractile muscles. Indeed, k_{fast} is decreased in dMNs to the level seen in WT vMNs (V.W. and R.A.B., unpublished observations). This is, perhaps, indicative that the dMNs require an instructive signal from their muscle targets in order to follow a different path of electrical development. Whether this path suppresses islet expression in dMNs remains to be determined. Significantly, vMNs were not affected in the Mhc1 mutant suggesting that repression of Sh-dependent Iₖ by Islet is independent of muscle derived input.

Why do motoneurons differ in their electrical properties and what is the functional implication? dMNs and vMNs receive differential synaptic drive (Baines et al., 2002) and innervate distinct muscle targets, dorsal obliques and ventral longitudinals, respectively (Landgraf et al., 1997). During larval crawling ventral muscles are recruited prior to dorsal muscles (Fox et al., 2006) to, probably, facilitate coordinated movement. Interestingly, synaptic strength, based on EJP amplitude, is largest between vMNs and their target muscles. While the precise underlying mechanism is unknown, pharmacology suggests that terminals of dMNs express a larger Sh-dependent K⁺ current compared to vMNs. This current disproportionately reduces presynaptic neurotransmitter release and hence regulates synaptic strength (Lee et al., 2008). Whether this alone...
Neuron
Islet Regulates Electrical Properties

can account for the delay of dorsal muscle contraction is not known. Differences in electrical properties, specifically delay to first spike, have also been observed between Drosophila motoneurons (Choi et al., 2004). While the precise reasons for these differences remain speculative, they are consistent with differential contribution to muscle activity that underlies locomotion in Drosophila larvae.

We can recapitulate the repressive effect of ectopic islet expression on Sh-mediated K⁺ current in body wall muscle. This is important for two reasons. First, it provides unequivocal support for the hypothesis that Islet is deterministic for expression of Sh in excitable cells, regardless of whether those cells are neurons or muscle. Second, body wall muscles are isopotential and do not therefore suffer from issues of space clamp (Broadie and Bate, 1993). Analysis of ionic currents in neurons can be complicated by such factors, which becomes more serious for analysis of those currents located further away from the cell body in the dendritic arbor. Hence electrophysiologically-tractable muscles may offer the possibility to derive a more complete understanding of the differential activity of codes of transcription factors on the regulation of ion channel development within the developing nervous system.

EXPERIMENTAL PROCEDURES

Fly Stocks

For larval collections, flies were transferred into laying pots and allowed to lay eggs onto grape juice agar plates. Laying pots were kept at 25°C and 18°C for motoneuron and muscle experiments, respectively. The following fly strains were used: Canton-S as wild-type (WT), islet mutant tup[ai-1]rd♂[1]hk[1]pr[1]/CyO (Cytact:GFP) (rebalanced from Bloomington 3586); Shaker mutant Sh[14] (Bloomington 3563, carries the KS133 mutation). The Shaker and islet mutations were combined in a double mutant Sh[14];tup[Is1-1]/CyO act::GFP. The 10 M KO (homozygous viable on the third chromosome) were used to express GFP in vMNs for in situ hybridization. GAL4RN2-0 (homozygous viable on the second chromosome) or GAL4RRa (homozygous viable on the 3rd chromosome) were used to express TeTxLC in all CNS neurons (Sweeney et al., 1995). GAL4Lim3 was used for muscles.

Embryo and Larval Dissection

Newly hatched larvae or late stage 17 embryos were dissected and central embryo and larval dissection (Baines and Bate, 1993). Analysis of ionic currents in neurons were accessed for electrophysiology as described byBaines and Bate (1993). Newly hatched larvae or late stage 17 embryos were dissected and central

Electrophysiology

Recordings were performed at room temperature (20°C to 22°C). Whole-cell recordings (current and voltage clamp) were achieved using borosilicate glass electrodes (GC100TF-10; Harvard Apparatus, Edenbridge, UK), fire-polished to resistances of between 15 - 20 MΩ for neurons and between 5 and 10 MΩ for muscles.

Neurons were identified based on their position within the ventral nerve cord. Neuron type was confirmed after recording by filling with 0.1% Alexa Fluor 488 hydrazide sodium salt (Invitrogen), which was included in the internal patch saline. Recordings were made using a Multiclamp 700B amplifier controlled by pClamp 10.2 (Molecular Devices, Sunnyvale, CA). Only neurons with an input resistance > 1 GΩ were accepted for analysis. Traces were sampled at 20 kHz and filtered at 2 kHz. The voltage-clamp protocols used to record total K⁺ currents were as follows: for neurons, from the resting potential of −60 mV neurons were hyperpolarized to −80 mV for 100 ms, the voltage was then stepped from −80 mV to +40 mV in increments of 0.10 mV for 60 ms. To isolate slow K⁺ currents a prepulse of −20 mV for 100 ms was used (Baines and Bate, 1998). For muscles a maintained holding potential of −60 mV was used and a −90 mV prepulse for 200 ms and voltage jumps of 0.20 mV increments were applied from −40 to −40 mV. Leak currents were subtracted off-line for central neuron recordings. For muscle recordings, however, on-line leak subtraction (P/4) was used. Recordings were done in at least four animals and at least eight neurons/muscles were recorded from in total for each experiment. Individual recordings were averaged, following normalization relative to cell capacitance, to produce one composite average representative of that group of recordings. Cell capacitance was determined by integrating the area under the capacity transients evoked by stepping from −60 to −90 mV (checked before and after recordings).

Membrane excitability (i.e., action potential firing) was determined using injection of depolarizing current (1, 2, 4, 6, 8, 10 pA/500 ms) from a maintained membrane potential (V_m) of −60 mV. V_m was maintained at −60 mV by injection of a small amount of hyperpolarizing current.

Solutions

Motoneuron Recordings

External saline for dissection and current clamp analysis of excitability consisted of the following (in mM): 135 NaCl, 5 KCl, 4 MgCl₂, 6 H₂O₃, 2 CaCl₂, 2 H₂O, 5 N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), 36 sucrose, pH 7.15. For isolation of total K⁺ currents 1 μM TTX (Alomone Labs, Jerusalem, Israel) was added to the external solution. For most recordings Ca²⁺-activated K⁺ currents were eliminated by adding Cd²⁺ (0.2 mM) to the saline. Sh-mediated K⁺ current was blocked using dendrotoxin (DTx, Sigma, 200 nM). Current clamp recordings were done in the presence of mecamylamine (1 mM, Sigma) to block endogenous cholinergic synaptic currents. Internal patch solution consisted of (in mM): 140 K⁺ gluconate, 2 MgCl₂, 6 H₂O, 2 EGTA, 5 KCl, and 20 HEPEs, pH 7.4.

Muscle Recordings

External saline (Stewart et al., 1994) for dissection and voltage-clamp analysis consisted of the following (in mM): 70 NaCl, 5 KCl, 0.1 CaCl₂, 20 MgCl₂, 6 H₂O₃, 10 NaHCO₃, 5 HEPEs, 115 sucrose, 5 trehalose (pH 7.2). The calcium concentration was kept low (0.1 mM) to prevent activation of Ca²⁺-dependent K⁺ currents. Internal patch saline was the same as for neurons.

In Situ Hybridization

In situ hybridization was performed as previously described (Choksi et al., 2006), using a hybridization temperature of 65°C. Five separate probes were generated to target an intron of Sh common to all splice isoforms (second intron of Sh-RB). The probes were equally mixed before use. The primers used to generate the RNA probes are as follows:

Sh_int1_FW (CTCTTTTCTTGGGATTGAAAGCACAT), Sh_int1_RVTT (CAGT AATAGCAGCTACTATTATATAGCACAAATTAGGAAGCAAGAT), Sh_int2_FW (TGGATCTGTTCAGTCTTTAT), Sh_int2_RVTT (CAGT AATAGCAGCTACTATTATATAGCACAAATTAGGAAGCAAGAT), Sh_int3_FW (ATTATCGAGTACTTTGCACTGATTTA), Sh_int3_RVTT (CAGT AATAGCAGCTACTATTATATAGCACAAATTAGGAAGCAAGAT), Sh_int4_FW (GCCAAGAGAAACGCTTGTTAAAATCT), Sh_int4_RVTT (CAGT AATAGCAGCTACTATTATATAGCACAAATTAGGAAGCAAGAT), Sh_int5_FW (AAAGGAATTCACGGACTAAACAT), Sh_int5_RVTT (CAGT AATAGCAGCTACTATTATATAGCACAAATTAGGAAGCAAGAT).

Immunohistochemistry was performed after the in situ protocol using an anti-Eve antibody (1:5,000; Frasch et al., 1987) or an anti-GFP antibody (1:2,000; Abcam ab6556) and developed using DAB.
ACKNOWLEDGMENTS

We would like to thank Drs. J. Jaynes, M. Fujioka, K. Koh, J. Skeath, and S. Thor for providing flies and Matthias Landgraf for comments on the manuscript. This study was funded by grants from the Wellcome Trust to R.A.B. (083837 and 090798) and AHB (programme grants 088055 and 092549). A.H.B. acknowledges the core funding provided by the Wellcome Trust (092096) and CRUK (C6946/A14492). Work on this project benefited from the Manchester Fly Facility, established through funds from the University of Manchester and the Wellcome Trust (087742).

Accepted: June 5, 2012
Published: August 22, 2012

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