NF-κB/Rel-Mediated Regulation of the Neural Fate in Drosophila

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Two distinct roles are described for Dorsal, Dif and Relish, the three NF-κB/Rel proteins of Drosophila, in the development of the peripheral nervous system. First, these factors regulate transcription of scute during the singling out of sensory organ precursors from clusters of cells expressing the proneural genes achaete and scute. This effect is possibly mediated through binding sites for NF-κB/Rel proteins in a regulatory module of the scute gene required for maintenance of scute expression in precursors as well as repression in cells surrounding precursors. Second, genetic evidence suggests that the receptor Toll-8, Relish, Dif and Dorsal, and the caspase Dredd pathway are active over the entire imaginal disc epithelium, but Toll-8 expression is excluded from sensory organ precursors. Relish promotes rapid turnover of transcripts of the target genes scute and ase sense through an indirect, post-transcriptional mechanism. We propose that this buffering of gene expression levels serves to keep the neuro-epithelium constantly poised for neurogenesis.

INTRODUCTION

The proneural genes of the achaete-scute (ac-sc) and ase families encode related transcription factors of the basic helix-loop-helix family that function as heterodimers together with the co-factor Daughterless/E2A/HEB/E2-2 [1]. The structure and function of these proteins is highly conserved throughout the animal kingdom. They are expressed in the neuro-epithelium during development and confer neural potential to cells through activation of neuronal precursor genes that regulate differentiation of neurons.

Single neuronal precursors are generated in a spaced array within domains of proneural gene expression through Notch-mediated lateral inhibition. This is a conserved process involving an indirect auto-regulatory loop whereby the proneural genes repress their own transcription. Cells chosen to be precursors sustain high threshold levels of expression and signal to the cells surrounding them causing them to gradually lose proneural gene expression [1]. Neuronal precursors move away from the neuro-epithelium and subsequently proneural genes are re-expressed. Successive waves of proneural gene expression thus allow the repeated generation of waves of neuronal precursors. This suggests that the neuro-epithelium is constantly poised to express the proneural genes in the absence of the inhibitory signal.

The array of sensory bristles on the Drosophila thorax is a useful paradigm for understanding the control of neuronal precursor development [2,3]. In the imaginal disc ac and sc are expressed in small clusters of cells from which one or two sensory organ precursors (SOP) are singled out [4,5,6]. High levels of Ac-Sc in cells chosen to be SOPs activate neuronal-specific genes such as ase (ase) and senseless (sens) [7,8,9,10,11,12,13].

Culi and Modolell [2] described a regulatory element in the sc gene, the SOP element, which mediates lateral inhibition. It drives auto-regulation of sc in the SOP itself and is probably a target for repression in the inhibited cells. The enhancer bears binding sites for Ac-Sc/Da (E boxes) and a potential site for repression by the E/pher bHLH proteins, targets of Notch signalling (N box). Three 3 boxes, motifs resembling the consensus binding sequence for transcription factors of the NF-κB/Rel family and a T-rich motif of unknown function were also found. Mutation of the 3 boxes in the sc SOP enhancer demonstrated a role for these sequences for maintenance of transcription in SOPs and for repression in inhibited cells [2]. This suggests a possible involvement of NF-κB/Rel proteins in SOP development. Three genes encoding NF-κB/Rel proteins are present in Drosophila: dorsal (dl), Dorsal related immunity factor (Dif) and Relish (Rel). All three are involved in innate immunity and dl is also required for dorso-ventral polarity of the embryo [14,15].

Toll-1 is known to initiate signalling leading to activation and nuclear translocation of the NF-κB/Rel proteins in Drosophila [15,16]. Here we demonstrate a dual role for the three NF-κB/Rel proteins and one of the receptors of the Toll family, Toll-8, in the regulation of neurogenesis. We find that the NF-κB/Rel proteins promote the neural fate in SOPs. Activation of sc via the 3 boxes in the SOP enhancer might be direct. In addition, Toll-8 and Relish act to maintain low levels of expression over most of the epithelium of the target genes sc, ase and sens, by promoting rapid turnover of mRNA. This is mediated by a post-transcriptional mechanism affecting both RNA stability and translation. It appears to involve a heptamer nucleotide motif present in the coding regions similar to that described for the indirect regulation by NF-κB of MyoD and Sox9 in mammalian cells [17]. We discuss the possibility that Relish regulates steady state levels of expression of genes required for the neuronal fate, so that the neuro-epithelium of the discs is constantly primed for neurogenesis.

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RESULTS

Mutants of Toll-8 and dorsal, Dif and Relish display ectopic bristles at 18°C

The three NF-κB/Rel proteins Dorsal, Relish and Dif are detected ubiquitously in the imaginal epithelium [2,18]. Since there are potential binding sites for these proteins in the sc promoter, we examined mutants of the three NF-κB/Rel genes for perturbations in the patterns of the large bristles (macrochaetes) of the notum. Flies homozygous for loss of function alleles of dl, Dif and Rel were found to display ectopic bristles on the notum in dorsocentral, scutellar and lateral regions (Figure 1B,C,D). The phenotype is in

Figure 1. Loss-of-function mutants of NFκB/Rel genes display both loss and gain of macrochaetes on the notum. (A), a schematic representation of a wild-type heminotum, with the medial domain shaded yellow and the lateral domain orange. Large grey circles represent the positions of the eleven macrochaetes found on each heminotum. (B–D), heminota of flies homozygous mutant for dorsal (dl4/dl1), Dif (Dif1/Dif1) and Relish (RelE20/RelE20); white arrows indicate ectopic bristles. Below, pie charts represent the percentage of heminota displaying ectopic bristles from a total of 200. Red sectors indicate the percentage with one ectopic macrochaete, yellow sectors two or more ectopic macrochaetes (p<0.001 when compared with wild type). (E, F), heminota of Ore-R and dl4/+ animals respectively, showing the lateral notum. Red arrows mark the pSA bristle, which is missing in a large fraction of dl4/+ (and also dl4/Df(2L)TW119, dl2 dif2 animals) (p<0.001). (G), the percentage of flies trans-heterozygous for various mutant combinations that display ectopic bristles (p<0.001).

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the form of one or more ectopic bristles per hemi-notum in a significant fraction of individuals for each genotype (typically 30% of heminota, n = 200 unless otherwise specified). It is observed in females reared at 18°C and is statistically significant (p<0.001). The phenotype is not seen at 25°C.

Animals heterozygous for these mutations also display bristle phenotypes at 18°C. While Rel^{P200}+/+ and Dif^{P12}/+ flies display ectopic dorsocentral bristles in the medial notum (26% and 8% respectively), bristles are missing in the lateral notum of Df^{P12}/+ flies (Figure 1G and E–F). In addition, while 32% of heminota of double heterozygous Dif^{P12}/++;/+/Rel^{P200} flies display ectopic medial bristles, this phenotype is suppressed by heterozygosity for Dl, so only 4% of heminota of animals triply heterozygous for Dl, Dif and Rel display ectopic medial bristles. We also recovered imagos triply homozygous mutant for Dl, Rel and Dif. Few animals of this genotype are viable, but the ten that did survive displayed a wild-type pattern of bristles. Taken together, the lack of observable phenotypes in the triple heterozygote and triple null suggests that the relative stoichiometric ratios between the three proteins are important in the final outcome on bristle patterning.

We also examined the activity of Toll-8, a member of the Toll-family of transmembrane receptors that initiate NF-κB/Rel signalling. A Gal4 insertion in the Toll-8 gene [19,20], MD806, was identified in a screen for insertion lines reporting gene expression in the adult thorax [21]. Toll-8 mutants were generated by imprecise excision of the transposon insertion in MD806. Here we employ Toll-8^{u}, a null allele (Figure S1). Toll-8^{u} mutants are viable and display a phenotype similar to Dl, Dif and Rel mutants: 65% of heminota in homozygous females display ectopic macrochaetes at 18°C.

Previous work has shown that Dorsal and Dif are sequestered in the cytoplasm by the IκB factor Cactus whereas Relish has its own IκB domain [15,22,23,24]. Two signalling pathways allowing nuclear translocation of these proteins are known to function in Drosophila. The first involves a phosphorylation cascade and the release of Dorsal and Dif from Cactus [15,23]. The second pathway leads to proteolytic cleavage of Relish by the caspase Dredd to remove the IκB domain [25,26]. Flies mutant for Dredd were found to display ectopic bristles at 18°C (not shown, 43% of heminota, n = 200). No involvement of cactus in bristle patterning could be detected from visible phenotypes in loss-of-function clones or in over-expression experiments (not shown).

Ectopic bristles were also observed in flies heterozygous for Toll-8 (10% heminota) and this trait was used to detect interacting components. The bristle phenotype of flies simultaneously heterozygous for either Dredd^{P112} and Toll-8^{u} (57% heminota), or Rel^{P200} and Toll-8^{u} (55% heminota), was significantly enhanced compared to the single heterozygotes (Dredd^{P112}/+ 12%, Rel^{P200}/+ 26%; Figure 1G). No interaction was detected between Toll-8^{u} and Dif^{P12} or between Toll-8^{u} and Dl^{u} (not shown). These results suggest a link between Toll-8 and NF-κB/Rel signalling via the Dredd pathway.

We conclude from these observations that NF-κB/Rel proteins are active in the imaginal epithelium and that the receptor Toll-8 also plays a role. The mutant phenotypes suggest a function for Dorsal, Dif and Relish in repression of the neural fate.

Over-expression experiments suggest that NF-κB/Rel proteins both promote and repress the neural fate

Ectopic bristles in the loss-of-function mutants are generally observed close to extant ones, suggesting that they arise from the proneural clusters of ac-sc expression. Therefore sca-Gal4, which is expressed in all proneural clusters on the notum, was used to over-express Rel, Dorsal and Dif.

Over-expression of full-length Rel at 25°C resulted in loss of bristles in the lateral notum and ectopic bristles in the medial notum (Figure 2A, B). Over-expression of Dorsal and Dif at 25°C resulted in a loss of bristles over much of the notum, although the loss is greater in the lateral notum (Figure 2C, 2G). However, over-expression of Dorsal at a lower temperature of 18°C also resulted in the generation of ectopic bristles on the medial notum in the dorsocentral and scutellar regions (Figure 2E). Note that the Gal4-UAS system used...

Figure 2. Over-expression of NF-κB/Rel proteins can result in both loss and gain of macrochaetes. sca-Gal4[537.4]Gal4 was used to over-express Relish (A, B) Dorsal (C–F) and Dif (G) in proneural clusters. This resulted in a loss of bristles in lateral regions (red arrows in B, D, F and G). Ectopic bristles were present in medial regions of animals after over-expression of Relish and Dorsal (white arrows in A, E).

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for these over-expression experiments is temperature-dependent: lower amounts of Gal4 and consequently less over-expressed Dorsal are synthesized at the lower temperature.

We conclude that the NF-κB/Rel proteins can promote or repress the neural fate in a region-specific manner and that differences are apparent between the activities of the three proteins.

**Toll-8 expression is down-regulated in sensory organ precursors**

Genetic interactions suggested a role for Toll-8 in NF-κB/Rel regulation of the neural fate, so we examined the expression pattern of Toll-8 in the larval wing disc. We employed MD806, a Gal4 insertion in the 5′UTR of Toll-8. Toll-8-Gal4 drives GFP reporter gene expression at very high levels in the lateral halves of the notum in discs at the third larval instar (Figure 3A,B). In situ hybridization with a Toll-8 probe revealed a very similar pattern of expression (Figure 3C), see also [27]. This covers the region where most of the bristles form, except the dorsocentral and scutellar bristles that arise just at the border where expression levels fall. Ectopic expression of sc using Toll-8-Gal4, resulted in the generation of ectopic bristles on the lateral notum as well as in the dorsocentral and scutellar regions (not shown). This suggests that Toll-8 is expressed in the medial notum but that the levels there are much lower. We also used Toll-8-Gal4 to drive UAS-Flp and obtain mitotic recombination with a cuticular marker f36a and found labelled cells over the entire notum in these flies (Figure 3D). This also indicates that Toll-8 is expressed in the medial notum (or was expressed there earlier in development). Overall these results suggest that Toll-8 activity is likely to be higher in the lateral notum. It is noteworthy that the bristle phenotypes seen after loss or gain of function of NF-κB/Rel gene activity display differences between the medial and lateral notum.

Significantly, levels of transcription of Toll-8 differ in the SOP with respect to cells surrounding it. Expression of Toll-8, visualized with Toll-8-Gal4, is gradually excluded from the SOPs as they arise (Figure 3B, B′). Exclusion from SOPs was confirmed by double labelling with the SOP reporter A101-lacZ (Figure 3A,
and is particularly well illustrated by the anterior postalar (aPA), posterior supraalar (pSA) bristles and sensilla trichoidea (tr1) precursors in the lateral notum. In late third instar larval discs, GFP expression fades in the early arising aPA precursor, which can be seen marked in red in Figure 3A, A'. By white prepupal stages, additional “holes” in the GFP expression pattern appear at positions corresponding to the later arising pSA and tr1 precursors (Figure 3B'). Ectopic SOPs also lose Toll-8 expression: mis-expression of sc using Toll-8-Gal4 generates several ectopic cells positive for the neuronal marker A101-lacZ, all of which lose GFP expression (not shown). Furthermore, whereas mis-expression of E(spl)m8 using the SOP-specific neur-Gal4 driver results in a complete loss of notal bristles (Figure 3E'), reinforcement the observation that Toll-8 expression is not retained within SOPs. Exclusion of Toll-8 from neural precursors in the embryo has been previously reported [20].

Expression of Toll-8 is non-uniform in the disc epithelium and transcription is down regulated in the SOPs. If Toll-8 is affecting the activity of one or more NF-κB/Rel proteins, this would suggest, by extrapolation, a similar discontinuity between the levels of NF-κB/Rel proteins in the SOP compared to the cells surrounding it.

NF-κB/Rel might recruited to the α boxes in the scute sensory organ precursor enhancer

Is sc a direct target of NF-κB/Rel signalling? The SOP enhancer of sc [sc-SOPE] [2] contains three consensus binding sites, α boxes, for NF-κB/Rel proteins (upper cartoon in Figure 4). To examine NF-κB/Rel-mediated transcriptional regulation of sc we employed sc-SOPE-lacZ, a construct containing the native SOPE (called SRV-lacZ in [2]) and sc-SOPEα3−lacZ, a construct in which one of the α boxes, α3, has been mutated. In wild-type discs sc-SOPE-lacZ is expressed in all SOPs (Fig 4A), but expression of sc-SOPEα3−lacZ is decreased in wild-type flies and after over-expression of RelVP16, but strongly increased in the presence of E(spl)m7VP16 whose activity does not rely on the same binding sites.

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is eliminated in all but four cells (Figure 4D) suggesting this motif is essential for activation [2].

To test for a direct role of NF-kB/Rel we expressed a chimeric protein containing full length Relish fused in-frame to the trans-activation domain of VP16, a potent transcriptional activator. The Gal4–UAS system was used to express Rel-VP16, but note that signalling is nevertheless required to process Rel-VP16 and allow nuclear access. We used Bx-Gal4, which drives expression in the wing pouch (visualized in Figure 4E, see below). The Bx expression domain overlaps an area of Toll-2 expression as well as proneural clusters of ac-sc expression from which sensilla of the wing margin, dorsal radius and third wing vein arise. ac-SOPE-lacZ staining was increased in Bx-Gal4>UAS-Rel-VP16 wing discs in a region in the centre of the Bx-Gal4 expression domain (Figure 4B). More cells are labelled than in wild-type discs (Figure 4A). In contrast, sc-SOPE3–lacZ is only very weakly expressed in Bx-Gal4>UAS-Rel-VP16 animals, although it can still be detected in more cells than in the control discs (Figure 4D,E). Therefore Rel-VP16 cannot efficiently activate ectopic transcription of the SOPE in the absence of the z3 site.

As a control for this experiment we employed a chimeric protein comprised of E(spl)m7, a transcription factor that would bind the intact E or N boxes in sc-SOPE3–lacZ and the activator VP16 [28]. Expression of this protein using Bx-Gal4 results in dramatic expression of sc-SOPE3–lacZ in most cells of the Bx expression domain (Figure 4F).

We noted that the ectopic cells with sc-SOPE-lacZ staining in Bx-Gal4>UAS-Rel-VP16 flies were close to the positions of extant SOPs (see in Figure 4A) suggesting that they have arisen from cells of the proneural clusters originally expressing ac-sc (Figure 4B). In contrast, when the same Bx-Gal4 driver is used to ectopically express Ace, a proneural transcription factor that would bind the E boxes in sc-SOPE-lacZ a dramatic up-regulation in many more cells throughout much of the Bx expression domain is seen (Figure 4C). These include cells outside the areas of ac-sc expression in the wild type. These results suggest that Rel-VP16 can only activate the SOPE in the cells that have high levels of Ac/Sc.

We suggest that NF-kB/Rel proteins might be recruited to the sc SOP enhancer via the appropriate z binding sites and could therefore directly activate or repress sc expression. Activation in the sensory organ precursor may require high levels of Ac/Sc.

scute, asense and senseless are ectopically transcribed in Relish mutants

Toll-2 is expressed throughout the epithelium and is excluded from SOPs. Further, loss-of-function mutants in Toll-2 and the NF-kB/Rel-encoding genes bear ectopic bristles. We therefore examined expression of the neuronal genes sc, ase and sens in these mutants. We chose to examine Toll-2 and RelE20 mutant discs because of the strong phenotype of RelE20 and the genetic interaction between these two mutants.

Two surprising features were observed. First, sc, ase and sens were seen to be expressed at higher levels in Toll-2 and RelE20 mutants than in wild-type control discs (Figure 5A–I). Samples for each genotype were fixed, processed and stained under identical conditions. Staining times were kept short to better visualize differences in staining intensity. Typically, within 5 minutes of staining, ase expression was apparent in the SOP cells of mutant discs, but not the wild-type controls. Second, the neuronal genes were expressed in significantly broader domains in the mutants. scute was seen to be expressed in enlarged proneural clusters (Figure 5A-C), asense and sens, whose expression is usually confined to the SOP, were globally de-repressed in the mutants and transcripts for these genes accumulated over much of the disc (Figure 5D–I). This is unexpected because only a few ectopic bristles develop in these mutants and ectopic expression of Ase and Sens in wild-type discs results in the generation of vast numbers of ectopic bristles. However, the ectopic transcripts were not mirrored by a corresponding accumulation of the protein products of these genes. We found that Sens protein is restricted to the SOP cells (Figure 5J-L). It appears that the de-repressed ase transcripts in Toll-2 and RelE20 mutants are not translated either (see Figure S2A–C). The distribution of other neuronal markers such as Achaete (Ac) (Figure S2D–F) or Hindsight (not shown) was also confined to the precursors in Toll-2 and RelE20 mutants.

We conclude that transcripts of sc, ase and sens are present both ectopically and at higher levels in Toll-2 and RelE20 mutants. The transcripts are not translated, however, and therefore do not result in the formation of large numbers of ectopic bristles.

The de-repression of target genes in Relish mutants is mediated by post-transcriptional effects on mRNA stability

To investigate whether the increased levels of sc, ase and sens mRNA present in mutant RelE20 discs are a result of increased transcription or greater transcript stability, we used a heterologous expression system based on Gal4–UAS activation. A recombinant chromosome bearing sca-Gal4 and UAS-GFP was placed in trans with chromosomes bearing UAS-ac, UAS-sc or UAS-ase, either with or without functional copies of the Rel gene. If the increase in mRNA in the Rel mutant is due to increased transcription, then no increase should be possible from a heterologous promoter. If, on the other hand, the transcripts are stabilized then mRNA from the heterologous promoter should accumulate in this experiment.

Reverse primers located within the 3′UTR of the ectopic transcripts (see Experimental Procedures) were used to prime cDNA synthesis and detect transcript levels. Transcripts specific for ac are not present at higher levels in the mutant, suggesting that this gene is not subject to regulation by NF-kB/Rel (Figure 6A). This result also indicates that loss of Rel activity does not have a significant impact on the expression of sca-Gal4. We detected enhanced levels of sc and ase transcripts in the RelE20 homozygous mutant background (Figure 6A). Unexpectedly, GFP transcripts were also present at much higher levels in the mutant (Figure 6A). GFP transcripts similarly result exclusively from activity of the Gal4–UAS driver and therefore cannot be subject to direct transcriptional regulation by Relish. We conclude that stability of the ectopic GFP, sc and ase mRNAs is increased in the mutant.

It has been shown that NF-kB can regulate target gene expression using post-transcriptional as well as transcriptional mechanisms. Loss of NF-kB signalling causes a decrease in endogenous mRNA levels of MyoD and Sex3 in C2C12 cells, an effect dependent on a heptamer motif, ACTACGAG, present in the coding sequence of both genes [17]. Examination of the coding sequences of sc, ase and GFP reveals the presence of a similar, but slightly modified motif, in which six of the seven nucleotides are conserved (ACTACA- Figure 6B). Two copies of the motif are present in the coding region of sc; a single copy is present in both the ase and sens genes. Notably the motif is present twice in the coding sequence of GFP. It is also present in other genes involved in patterning the notum (Figure 6B). Interestingly this sequence is not found in ac, a fact consistent with the lack of transcript accumulation in ac mutants.

If the heptamer motif does indeed mediate rapid turnover of sc, ase and sens transcripts, then mRNA of mutants devoid of this motif should be stabilized. Examination of existing sc mutants led us to
one such mutant: seHu$^{ts}$. seHu$^{ts}$ carries a complete copia element within the sc coding sequence causing a truncated transcript [29]. We have located the copia insertion to position 904bp within the sc gene near the end of the coding region. The aberrant transcripts would therefore retain only one of the heptamer motifs (Site M2, Figure 6D). Interestingly, seHu$^{ts}$ mutant flies display one or two ectopic bristles (Figure 6C,E) and furthermore a 5-fold increase in sc transcript levels [29]. If Relish acts via the heptamer motif, then double mutant seHu$^{ts}$ RelE20 flies may be expected to display a more extreme phenotype than that of seHu$^{ts}$. Indeed seHu$^{ts}$ RelE20 flies have more bristles than seHu$^{ts}$ Rel$^{+}$ animals (Figure 6E). seHu$^{ts}$ RelE20 flies also have more bristles than RelE20 flies suggesting that Relish is not the only NF-κB/Rel factor involved in this phenotype. These results suggest a possible role of the heptamer sequence in transcript stability.

We conclude that Relish acts on se and as through a post-transcriptional mechanism inducing rapid mRNA turnover. The seHu$^{ts}$ phenotype is consistent with a role for a heptamer motif in
Figure 6. Transcript stability in Relish mutants correlates with the presence of a heptamer sequence in the coding regions of target genes. (A), levels of heterologous (UAS) achaete, scute, asense and GFP transcripts driven by sca-Gal4 in wild-type (left column) and RelE20 (right column) flies. The ribosomal RNA RP49 was used as a loading control. Levels of scute and asense but not ac transcripts are elevated in the mutant. In addition levels of GFP transcript are elevated. (B), a sequence motif similar to that described in Sox9 and MyoD (ACTAGA) is present in scute, asense, senseless and a number of other genes involved in patterning the notum. The central five core element nucleotides, CTACA, are conserved in all cases. The 3'-most nucleotide, G, is replaced in most cases by a T or an A. The 5'-most nucleotide, A, is replaced by a G in three cases (shown in grey). The final base, G, is mostly substituted by A or T. (C), the sequence of scHwUa is presented. Transcription stops within the copia element whose sequence is given in red. The two MyoD motifs are outlined in grey; one, M2, is predicted to be absent from the truncated transcript. (D), a photograph of a scHwUa mutant fly showing the presence of an ectopic dorsocentral bristle (white arrow). (E), pie charts representing the percentage of heminota displaying ectopic bristles from a total of 200. Red sectors indicate the percentage with one ectopic macrochaete, yellow sectors two or more ectopic macrochaetes (p>0.001 when compared with wild type).

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the transcribed regions of these genes that is similar to that regulating transcript stability in MyoD and Sox9 [17].

DISCUSSION

**Dual regulation of neural genes by NF-κB/Rel**

Our results suggest a dual role for the NF-κB/Rel proteins of *Drosophila* in the formation of SOPs (Figure 7). First, they could be recruited directly to the *sc* promoter and regulate transcription. The SOP enhancer of *sc*, required for auto-regulation of *sc* in the SOPs, contains z boxes, consensus sequences for NF-κB/Rel [2]. Culi and Modolell (1998) obtained evidence for a role of these sequences in both activation and repression of *sc*. Expression of Rel-VP16, a potent transcriptional activator form of Relish, is able to ectopically activate a reporter gene containing the intact *sc* SOP enhancer but not one in which the z3 box is mutated. So activation in this experimental situation requires the presence of an intact z3 site. The experiment does not rule out indirect effects, so further work is required to verify whether activation is direct. We suggest the NF-κB/Rel proteins participate in activation and repression of transcription of *sc*, a hypothesis consistent with *dl*, *Dif* and *Rel* mutant phenotypes of additional as well as missing bristles. Second, we describe an unexpected role of Rel in mRNA turnover of *sc*, *ase* and *sens*, neuronal genes required to specify and/or maintain the neuronal fate of SOP cells [8,9,10,11,12,13]. In *Rel* mutants, transcripts of *sc*, *ase* and *sens* accumulate due to increased transcript stability. Therefore in the wild type, Relish promotes rapid mRNA turnover, presumably indirectly through an unidentified transcriptional target. A similar phenotype is observed in *Toll-8* mutants, which furthermore, interact genetically with *Rel* mutants. Transcripts for *Rel* are reduced in the *Toll-8* mutant suggesting a role for Toll-8 in maintaining the levels of *Rel* transcript (Figure S3). This might be the reason for the genetic interaction.

A number of differences are apparent between mutants of the three NF-κB/Rel-encoding genes of *Drosophila*. Mutants triply homo- or hetero-zygous have a normal complement of bristles, while single homo- or hetero-zygous animals have either additional or missing bristles. This suggests possible opposing functions for these genes. Furthermore bristle phenotypes due to loss or gain of function differ in detail between the three mutants. Together these results point to the importance of the stoichiometric relationships between the three NF-κB/Rel proteins and raise the possibility that different Dorsal/Dif/Relish homo- or hetero-dimers may have distinct binding sites and therefore different targets [30,31,32]. This merits further investigation.

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**Figure 7. A model for the role of NF-κB proteins in buffering levels of the neural genes scute and asense.** Epidermal cells express high levels of Toll-8, which promotes high nuclear levels of NF-κB/Rel. Relish activates the transcription of an unknown target gene, whose activity results in the degradation of *scute* and *asense* transcripts. This activity extends throughout the neuro-epithelium of the disc. Once mature precursors are chosen, they stop expressing Toll-8 and low levels of the NF-κB/Rel proteins might be recruited to the *scute* SOPE, where they would synergize with the bHLH protein Scute to activate transcription of high levels of *scute*.

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The ratio of proneural to NF-κB/Rel proteins might determine activation or repression of transcription of scute

If NF-κB/Rel proteins both activate and repress sc, then they are expected to activate in SOP cells and repress in cells of the proneural clusters not chosen to be SOPs. We discuss two possible ways that this could occur. First, activation in the SOP may rely on high levels of proneural protein and low levels of NF-κB/Rel protein; conversely repression may require low levels of proneural and high levels of NF-κB/Rel protein. Notch-mediated lateral inhibition results in high levels of Sc in the SOP and lower levels in surrounding cells. Toll-8 expression is excluded from SOP cells suggesting, that if Toll-8 affects NF-κB/Rel activity, there would be lower levels of NF-κB/Rel in SOPs. NF-κB has been shown to activate transcription even without stimulus if IκB levels are low enough to allow NF-κB-dependent gene expression in the basal state [33]. Interestingly, it has been shown that low levels of Dorsal can act synergistically with bHLH proteins to activate target genes in the embryo [34]. This depends on direct association of Dorsal and bHLH proteins and cooperative binding to closely linked binding sites for the two respective proteins [34,35]. Furthermore these authors demonstrated cooperative binding for Sce and Dorsal. In the sc enhancer of the z boxes is indeed close to an E box, so perhaps high levels of Sc and low levels of NF-κB/Rel combine to activate transcription in the SOP. Two observations are consistent with this hypothesis: Rel-VP16 was able to ectopically activate sc-SOPE-lacZ only at sites where ac and sc are expressed and, after over-expression of NF-κB/Rel proteins, bristles are generally missing on the lateral notum (where Toll-8 levels are high), whereas ectopic bristles are found on the medial notum (where Toll-8 levels are low).

A second means by which NF-κB/Rel proteins could act differently in SOP and in non-SOP cells, may be the presence/absence of co-factors. It has been shown that Dorsal can be converted from an activator to a repressor by association with the co-repressor Groucho [36]. This bi-functionality is attributable to the fact that DI only weakly interacts with Groucho (37). During embryogenesis both Cut and Deadringer bind an AT-rich silencer sequence, AT2, present in target genes of Dorsal and both Dorsal and Deadringer bind the co-repressor Groucho and recruit it to DNA [38]. A similar AT-rich sequence (the β box) is present in the sc enhancer [2]. Furthermore repression of sc by the E(spl) proteins, targets of Notch signalling in non-SOP cells, is already known to require the activity of Groucho [2,39,40,41].

Toll-8 and Relish promote rapid turnover of transcripts of neuronal genes

Transcripts for sc, ase and sens (and GFP) accumulate in Rel and Toll-8 mutants as a result of increased transcript stability. Transcription stability correlates with the presence of a six or seven nucleotide motif in the transcribed sequence of these genes. The motif is present in sc, ase and sens, but not ac the transcription of which is unaffected in Rel mutants. The motif is almost identical to the heptamer in MyoD and Sox9 that is associated with transcript stability after inhibition of NF-κB/Rel signalling in C2C12 cells [17]. A sc mutant with a truncated sc transcript lacking one of the two motifs present in the coding sequence of this gene, has a phenotype similar to Rel and Toll-8 mutants and an increase in sc mRNA. Rabinow et al [1993] suggested that increased stability of the transcripts rather than increased transcription underlies this phenotype. We note the presence of the heptamer in a number of genes involved in sensory organ patterning suggesting possible regulation by NF-κB/Rel of a battery of genes in the imaginal epithelium. A similar motif is present in other vertebrate targets of NF-κB/Rel [42]. Post-transcriptional regulation of target genes by NF-κB/Rel could therefore be an ancient feature common to Drosophila and mammals and possibly even jellyfish. Sitcheran et al [17] suggest that an unknown factor, presumably a transcriptional target of NF-κB/Rel, regulates messenger turnover through association with this sequence. In Rel and Toll-8 mutants the accumulated transcripts are not translated. This must be an effect of the mutants because ectopic expression in wild-type flies allows translation and ectopic bristle formation.

Promotion of a rapid turnover of transcripts of neuronal genes presumably does not take place in the SOPs where high levels of the protein products of these genes are required. Accordingly Toll-8 expression is extinguished in the SOPs after their formation. Factors specific to the SOP presumably allow translation of the transcripts. We therefore suggest that high levels of Relish provided by Toll-8 in non-SOP cells might be required for post-transcriptional regulation of neuronal genes.

Maintenance of steady state levels of gene expression by NFκB/Rel may keep the neuro-epithelium primed for neurogenesis

In wild-type animals expression of neuronal precursor genes such as sens and ase is restricted to SOPs where they are activated by high levels of Ac and Sc [9,10,11]. Our results suggest that they are in fact expressed over the entire neuro-epithelium but that mRNA turnover is rapid due to NF-κB/Rel action. Activation of ac and sc in proneural clusters would counteract the effects of NF-κB/Rel to allow selection of SOPs. After selection of SOPs for the large sensory bristles is finished, Toll-8 expression is maintained in the epithelium, suggesting that high levels of NF-κB/Rel are still required for continued transcript turnover. Continuous buffering of neuronal gene expression presumably continues until the next round of neurogenesis that takes place after pupariation when precursors for the small bristles form. Therefore we hypothesize that NF-κB/Rel plays a subtle role in maintaining steady state levels of expression of many genes required for neural development. The maintenance of low levels of expression of neuronal genes would keep the tissue poised for neurogenesis that takes place in repeated rounds. Perhaps low levels of expression of neuronal genes are characteristic of neuro-epithelia in general.

Conclusions

Our hypothesis concerning the dual role of NF-κB/Rel in neurogenesis in Drosophila is as follows. The neuro-epithelium of the imaginal discs expresses neuronal genes. Prior to development of SOPs, high levels of Toll-8 maintain high levels of Rel and result in nuclear accumulation of NF-κB/Rel. Through an unknown transcriptional target(s), Relish promotes rapid turnover of neuronal transcripts by a post-transcriptional mechanism. This might be mediated by a specific sequence in the coding regions of target genes. Activation of ac and sc in proneural clusters by regulatory proteins of the notal prepattern counteracts the effects of Relish. After singling out of SOPs by Notch-mediated lateral inhibition, Toll-8 expression ceases in the SOPs. Reduced levels of signal uncover a trans-activator function for NF-κB/Rel that, synergistically with Sc, helps to maintain high levels of sc expression in the SOP, possibly through direct binding to consensus sequences in the sc enhancer. The NF-κB/Rel proteins may also directly repress sc in non-SOP cells of the proneural clusters. It remains to be seen to what extent each of the three proteins participates in these two processes.
MATERIALS AND METHODS

**Drosophila culture and stocks**

Flies were maintained on standard cornmeal-agar medium at 18°C and Oregon-R was used as a control. Strains used were: dl11 Gt(1B) Cyo, dl11 prl cn1 wxwxt bw/CyoO, Df(2R) H1/ Cyo-GFP, Df(2R) TW119/Cyo, RelE20/TM6b (other alleles of Rel (RelF13, RelF15, and RelF16) display a similar phenotype, not shown), Toll-8*/TM6b, Dredd+/sc [HwuUa] and sc [HwuUa]; RelE20. Strains used for NFκB/Rel misexpression were sc [537.4]-Gal4>GFP, Bx[MS1096] Gal4, pnr[MD237] Gal4> GFP, Mae-UAS.6.11- lacZ, Mae-UAS.6.11-lacZ, UAS-HA-Rel and UAS-HA-VP16-Rel. lacZ reporter strains were used as sc-SOPE-lacZ, sc-SOPE2–3’-lacZ and neur[Al101]-lacZ. Other strains used for ectopic expression were UAS-Esplm8, UAS-Esplm7ACT, UAS-ac, UAS-sc and UAS-HAase. The genotype y [5Sa abx >r] > Gal4; UAS Flp; Toll-8-Gal4 was used to ascertain the extent of Toll-8 terminal HA-VP16-Rel fusion.

**Mutagenesis**

The fly strain MD806 [21] was identified as an insertion in the 5‘-UTR of Toll-8 by plasmd rescue and used to generate the Toll-8 deletion by standard P-element excision. Several P[w+] strains were established and the extents of the deletions were confirmed by PCR. Primer pair U6 (CTTCAGCCAGCCGACCTGAT) and L10 (GGTGACAAGCGGAGAGCATTG) was used to determine the precise breakpoints of the R5A strain and primer pair U5 (AGCC-TCTAGAAAGGCGCAGTG) and L5 (AAGATTCTCGGGGCGCAGTAC) was used to generate a probe for in situ hybridization.

**Bristle scoring**

All mutant chromosomes used for the scoring were placed over either CyO-GFP (Chr II) or TM6b,Tb (Chr III) chromosomes and animals of the appropriate genotypes were Non-GFP or Cyo-GFP, Df(2L)TW119/Cyo, RelE20/TM6b (other alleles of Rel (RelF13, RelF15, and RelF16) display a similar phenotype, not shown), Toll-8*/TM6b, Dredd+/sc [HwuUa] and sc [HwuUa]; RelE20. Strains used for NFκB/Rel misexpression were sc [537.4]-Gal4>GFP, Bx[MS1096] Gal4, pnr[MD237] Gal4> GFP, Mae-UAS.6.11- lacZ, Mae-UAS.6.11-lacZ, UAS-HA-Rel and UAS-HA-VP16-Rel. lacZ reporter strains were used as sc-SOPE-lacZ, sc-SOPE2–3’-lacZ and neur[Al101]-lacZ. Other strains used for ectopic expression were UAS-Esplm8, UAS-Esplm7ACT, UAS-ac, UAS-sc and UAS-HAase. The genotype y [5Sa abx >r] > Gal4; UAS Flp; Toll-8-Gal4 was used to ascertain the extent of Toll-8 expression. See FlyBase for a description of mutants (http://flybase.bio.indiana.edu/).

UAS-constrasts for ectopic expression of haemagglutinin (HA)-tagged Rel were generated by standard techniques. The VP16 TA domain was PCR-amplified from a fly bearing the Ulhx-VP16 fusion, and cloned in frame into pHA-Rel to generate an N-terminal HA-VP16-Rel fusion.

**Mutagenesis**

The fly strain MD806 [21] was identified as an insertion in the 5‘-UTR of Toll-8 by plasmd rescue and used to generate the Toll-8 deletion by standard P-element excision. Several P[w+] strains were established and the extents of the deletions were confirmed by PCR. Primer pair U6 (CTTCAGCCAGCCGACCTGAT) and L10 (GGTGACAAGCGGAGAGCATTG) was used to determine the precise breakpoints of the R5A strain and primer pair U5 (AGCC-TCTAGAAAGGCGCAGTG) and L5 (AAGATTCTCGGGGCGCAGTAC) was used to generate a probe for in situ hybridization.

**Bristle scoring**

All mutant chromosomes used for the scoring were placed over either CyO-GFP (Chr II) or TM6b,Tb (Chr III) chromosomes and animals of the appropriate genotypes were Non-GFP or Non-Tb individuals. Triple null animals were selected as Non-GFP, Non-Tb animals and confirmed by single-fly genomic PCR using primers specific for doral (D1A AGGGTCCAGCACCGTGTATG, DBzet1 TGCTTGTGGACATCCTGCTG, Df/b DIFFI CCGACCATGAGTTGGAATGG, DEFRI GATCTCGGTGCTTCTGTAG), Rel (Rel5 CCAACCTTAATCTCCGAG, Rel9 AATATCGGCTGTGCGAGCG). The unrelated third chromosome gene del(3L) (Deil GATCTGAATGACATGCC, Dei2 CCGCCTGAATGATTTGCG) was used as an internal control.

Females grown at 18°C were examined for ectopic bristles and 200 hemihoraces were scored for each genotype, except dl1/dl1 (146 hemihoraces). Statistical analysis was performed using Student’s T-test. Pe charts were generated using Microsoft Excel.

**Histochemistry and immunolabelling**

Primary antibodies used included Anti-GFP-Alexa 488 (Molecular Probes), anti-Senseless (Bellen lab), anti-β-filag 40-1a, anti-Achaete and Anti-Hindsight (DSHB). Secondary antibodies coupled to Alexa 488, Alexa 546 and Alexa 647 (Molecular Probes) were used. Fluorescence images were taken with a Leica microscope using FW4000 software. Images were processed with Adobe Photoshop.

**In situ hybridization**

Samples were fixed overnight in 4% formaldehyde and then processed for in situ hybridization using standard techniques. Full-length cDNA clones of sc and ase and a 1kb PCR-amplified fragment of sens were used as templates to generate DIG-labelled RNA probes. All samples were processed in identical fashion. The experiments were repeated at least 4 times.

**Ectopic expression assay**

sc [537.4]-Gal4>GFP, UAS-ac, UAS-sc and UAS-HA-ase second-chromosome insert stocks were established individually with RelE20 on the third chromosome. Gal4>UAS crosses were set up simultaneously as the Gal4>UAS, RelE20 crosses. 10 wandering larvae were collected for each pair and processed for semi-quantitative RT-PCR. RNA was extracted using TRizol reagent (Life Technologies) and 200ng of this RNA was used for reverse transcription using gene-specific reverse primers and SuperScript TM II RNaseH− Reverse Transcriptase (Life Technologies). Total RNA was treated with DNase I to remove possible contaminating genomic DNA. Transcripts of the ribosomal protein RP49 were used as RNA loading controls. PCR amplification using gene-specific forward and reverse primers (25 cycles) was performed using Taq DNA polymerase (Roche) on an Eppendorf Mastercycler. Since the amplified transcripts represented a mixture of endogenous and ectopic RNA, we repeated the reverse priming with primers located in the SV40 tail of the UAS constructs. Subsequent PCR using gene-specific primers then resulted in the exclusive amplification of ectopic (UAS) transcripts.

Primers used were SV401 CGGTAGGTAGTTGTTGTC, SV402 GGGGCCTTCACAAAGATC, RP49-5’ ATGACCATTCCCCCGAGCATAC, RP49-3’ TTACCTGTTCCTTTGTTGAG, Ac1 GCCAGAGAAGATTTCCCATG, Ac1r GTTGT TTTCAGGTCTGTCTGTCG, Scf1 CATGTGATCTGAGTCGCT, Scf1 ACTGTGACTGCTGGACTC, Asef3 GGCACAACCAGCAGAATC, Asef3r CTCTTGTGAATCCGGGAAG, Gfp1r GCCAGAGAACCTTTTACTC, Gfp2r CCCTTTGTTAAATAAGATCGAG

The scute gene from the HueUa allele was amplified using primers Scf1 (CCATGTGATCTGAGTGCT) and Copiar1 (TGTTGAGTTGTCAGCT). This PCR fragment was sequenced and the exact breakpoint of the copia insertion was established.

**SUPPORTING INFORMATION**

**Figure S1** Recovery of a null allele of Toll-8. The insertion in MD806 maps 160bp upstream of the site of initiation of transcription and 640bp upstream of the translation start site and so was used to generate Toll-8 mutants by imprecise excision (A), several mutant lines were established and the extents of the deletions were confirmed by genomic PCR. Several primer pairs spanning the length of the Toll-8 gene and its upstream sequences were used to test the R5A strain and a 1.8kb U6L10 fragment normally spanning 4kb in wild-type flies was cloned and sequenced to confirm the extent of the deletion. This mutant strain was found to have a deletion of 2.24kb in the Toll-8 gene and was renamed Toll-8*. (B), Toll-8* flies lack detectable transcript as judged by in situ hybridization with a U5L3 probe (C) and are predicted to be protein-null due to the absence of the usual translational start site. Found at: doi:10.1371/journal.pone.0001178.s001 (0.29 MB TIF)

**Figure S2** The protein products of neuronal precursor genes are confined to sensory organ precursors in Toll-8 and Relish mutants. The neuronal-specific reporter gene sc-SOPE, lacZ is found in a normal distribution of precursors in wild type (A), Toll-8* (B)
and Rel (C) mutants. sc-SOPE-lacZ contains three E-boxes, which are binding sites for bHLH proteins such as Scute and Ason. Over-expression of ase in wild-type animals leads to ectopic expression of sc-SOPE-lacZ presumably due to generation of functional Ase protein (see Figure 3C). However, the ectopic expression of ase observed in the NF-κB mutants does not generate a corresponding global overexpression of sc-SOPE-lacZ (A–C), leading to the conclusion that Ase function in these discs is still confined to the SOPs. Staining with an antibody against Achaete shows a pattern of expression in the normal numbers and positions of the bristle precursors in wild type (D), Toll-8 (E) and Rel (F) mutants.

Found at: doi:10.1371/journal.pone.0001178.s002 (0.63 MB TIF)

Figure S3 Toll-8 is required to maintain transcription of Relish. Relish transcripts are reduced in Toll-8 homozygous (A), so Toll-8 may affect transcription of Relish. However, over-expression of Toll-8 in Gal4-C765>UAS-Toll-8 does not lead to a concomitant elevation in levels of Relish transcripts (B), indicating that the role of Toll-8 may be confined to maintenance of Relish transcript levels.

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Author Contributions

Conceived and designed the experiments: PS SA DP. Performed the experiments: SA DP MC AB KG CG. Analyzed the data: SA. Wrote the paper: PS SA.
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