Box 1. Key symposium outcomes

- Molecular definition of influx–efflux and secretory mechanisms
- Neuroprotective role of choroid plexus efflux of xenobiotics
- Molecular-ultrastructural correlations in tight junctions
- Choroid plexuses and CSF as major permeability route in developing brain
- Barriers remain a significant problem for drug entry into brain, but new approaches are in sight
- A high-tech approach to clinical monitoring pays off in understanding brain function in the critically ill

spinal cord. Habgood pointed out that carrier mechanisms, such as Penetratin, might provide an effective barrier-passing mechanism if they are targeted to cerebral endothelial cells. Tony Hughes (University of Melbourne, Australia) dealt with the use of a naturally occurring system for brain targeting (BDNF peptide fragments), and David Begley (King's College London, UK) described the *in vitro* assay of P-glycoprotein substrates and inhibitors as a means of screening compounds for their ability to enter or promote entry into the brain.

Box 1 outlines major outstanding topics in the field that emerged from discussions about the present state of blood–brain barrier research. The abstracts for all papers can be found at http://www.blood-brain-barrier-tasmania.org/ or http://www.lyon151.inserm.fr/choroid_plexus/.

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Techniques & Applications

Combinatorial RNAi: a method for evaluating the functions of gene families in *Drosophila*

Aloisia Schmid, Benno Schindelholz and Kai Zinn

Individual members of gene families often have partially redundant functions during nervous system development, making conventional genetic analysis problematic. Here we review experiments showing that several genes can be silenced together by injection of double-stranded RNAs into wild-type *Drosophila* embryos. By dye-labeling single neuroblasts in injected embryos, the effects of multigene silencing on individual CNS axon pathways can now be examined.

The genomes of model organisms encode thousands of proteins that are not affected by mutant alleles isolated in conventional genetic screens. How can the functions of such proteins be addressed? In the nematode *Caenorhabditis elegans*, a powerful approach to this problem has been developed in which synthetic double-stranded (ds) RNAs corresponding to specific genes are used to generate phenotypes in the F1 progeny of worms injected or fed with dsRNA [1,2]. This general process of post-transcriptional gene silencing by dsRNA is known as RNA inhibition (RNAi) and has recently been used for mass screens of gene function, allowing the phenotypes of more than 2000 genes to be determined [3,4].

Shortly after the RNAi technique was described in worms, it was demonstrated that RNAi could also work in *Drosophila* embryos [5,6]. With the completion of the *Drosophila* genome sequence, it became possible to perform RNAi experiments for all of its ~14 000 predicted genes.

We were interested in using RNAi to evaluate how genes regulate axon guidance and synaptogenesis during fly development, and wished to determine whether it could help solve some of the problems that are encountered in conventional genetic analyses of this problem. One such problem is genetic redundancy. Our group has studied this for a family of neural receptor tyrosine phosphatases (RPTPs; Box 1). Five RPTPs have been shown to regulate axon guidance, and single mutations in the genes encoding three of these (*Dlar*, *Ptp52F* and *Ptp69D*) confer lethality. Null mutations in the other two genes (*Ptp10D* and *Ptp99A*) however, produce no detectable embryonic phenotypes, and offspring are viable and fertile. The RPTPs have partially redundant functions, so that phenotypes are observed for *Ptp10D* and *Ptp99A* mutations only when they are combined with mutations in other *Rptp* genes. *Dlar*, *Ptp52F* and *Ptp69D* phenotypes are changed when mutations in these genes are combined with other *Rptp* mutations [7] (reviewed in Refs [8,9]).

Combinatorial RNAi as a tool for functional genomics

These and many other results indicate that: (1) when a new member of a gene family is identified in the genome sequence, it is not clear what phenotype to

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Box 1. Useful websites

- Carthew lab protocols on RNAi in *Drosophila* embryos (http://www.pitt.edu/~carthew/manual/RNAi_Protocol.html)
- A complete description of *Drosophila* NB lineages, including 3D movies (http://www.uoneuro.uoregon.edu/doelab)
- Images and descriptions of *Drosophila* neuromuscular innervation, and information about the functions of RPTPs in pathfinding (http://www.its.caltech.edu/~zinnlab/links.html)
- Andrew Fire's lab manual on dsRNA injection (http://www.ciwemb.edu/pages/firelab.html)

expect when searching for mutant alleles. Will a mutation produce lethality, and will it have a detectable anatomical phenotype in embryos? RNAi allows rapid determination of what phenotype to expect for a mutant gene before searching for mutations; (2) because of genetic redundancy, single mutants lacking one member of a gene family often do not have strong phenotypes. To define the functions of members of such families, it is often necessary to analyze multiply mutant combinations. Combinatorial RNAi facilitates experiments in which nervous system phenotypes are examined using antibody staining, because it eliminates the necessity to construct multiply mutant stocks and to identify embryos of the desired genotype among collections from such stocks; (3) owing to the limited number of antibody markers for specific CNS axonal pathways, selective visualization of the pathways most affected by loss of a particular protein is often not possible. By using the technique of DiI labeling of single neuroblast (NB) lineages, every axon pathway in the embryo is accessible (Box 1) [10]. However, DiI labeling on multiply mutant genotypes is difficult, because only a small fraction of embryos in a collection will be mutant for all the genes in question, and these mutant embryos cannot be identified at the stages during which labeling is performed. Combinatorial RNAi methods circumvent this problem.

To address these issues, we evaluated whether we could silence several members of a gene family at once using dsRNA injection. We determined conditions under which injection of multiple dsRNAs would produce specific phenotypes with a high penetrance without generating nonspecific toxicity [9]. We combined dsRNA injection and DiI labeling, establishing conditions under which we could inject wild-type embryos with one or more dsRNAs and later label NB lineages in the same embryos (Figs 1,2). These techniques are briefly described below, and further information and detailed protocols are available on request from A.S.

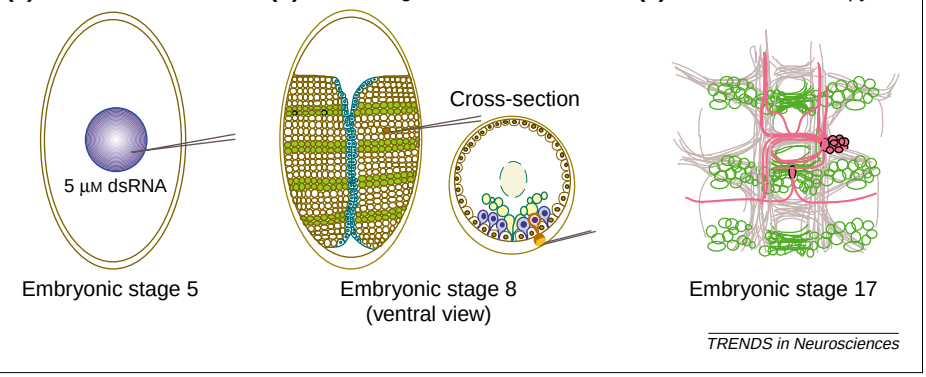

Fig. 1. Combinatorial loss of gene function can be analyzed at the single cell level using RNAi in *Drosophila* embryos. (a) Newly hatched eggs are aged to the blastoderm stage (stage 5) for dsRNA injection (blue drop). (b) Injected eggs are aged to stage 8 for DiI injection (red drops) of single neuroblasts (NBs). The green stripes represent green fluorescent protein (GFP) driven from the engrailed promoter. *en*-GFP serves as an *in vivo* positional marker and an internal control, being unaffected by *Rptp* gene silencing. (c) Embryos develop to stage 17, at which time DiI-labeled NB progeny neurons and glia are visualized by confocal microscopy. A cartoon of a NB 4-1 clone is shown here. Cell bodies and axons of neuronal progeny are indicated in purple, the axon scaffold in gray, and the *en*-GFP stripes as green circles. Embryos not labeled with DiI can also be dissected at stage 17 and stained using immunocytochemistry.

We have already used these methods to illuminate aspects of RPTP function [7,9]. First, we identified a new RPTP gene that was expressed at low levels. This gene, *Ptp52F*, lay within a region that had been subjected to an EMS screen for lethals, but there were many complementation groups within this interval and we had no way of knowing which, if any, of these corresponded to *Ptp52F*. We injected embryos with *Ptp52F* dsRNA and observed that the SNa motor nerve failed to bifurcate and the injected embryos did not survive. (As a control, we injected *Dlar* dsRNA, which produced no SNa phenotypes but caused the predicted ISNb pathfinding errors at the appropriate penetrance.) We then screened an allele from each of the lethal complementation groups within the 52F region and found one that produced SNa bifurcation failures. Sequencing then showed that three alleles within this complementation group had missense mutations in the *Ptp52F* coding region [7].

Second, we had defined the late phenotypes of quadruple mutants lacking four of the RPTPs [9,12] (Fig. 2), but because it was impossible to identify these embryos (1/128) at earlier stages by anti-RPTP antibody staining, we could not investigate pioneer neuron pathways in these mutants. By injecting wild-type embryos with dsRNAs for all four RPTPs, we were able to define how the pioneer pathways form when all four proteins are missing [9].

Technical considerations

In their initial work, Fire *et al.* [1] assayed the ability of RNAi to inhibit *unc-22A* function in *C. elegans*. They injected single (sense and antisense) strands of *unc-22A* RNA into the gonads of *C. elegans* adults, and assayed their F1 progeny for twitching behaviors indicative of a loss of *unc-22A* function. They discovered that injecting more than 3.6 million molecules of *unc-22A* sense or antisense RNA produced phenotypes in only 1–10% of the F1 progeny. Injecting less than 0.8% of that amount of *unc-22A* dsRNA however (i.e. 'several molecules per cell' [1]), produced 30% phenotypic penetrance in F1 progeny (see link in Box 1).

In our examination of the efficacy of RNAi in *Drosophila*, we focused on whether combinations of molecules could be used as effectively as single molecules. Combinatorial RNAi with two dsRNAs

had been demonstrated to work in *C. elegans* [12]. Because we already knew the phenotypes associated with the loss of any two,three or four of the RPTPs [7,9,12], we injected combinations of *Rptp* dsRNAs together at various concentrations. Initial experiments, in which we used relatively low concentrations of dsRNA for injection, produced embryos with weak and variable phenotypes.

We then performed optimization experiments, injecting embryos with a mixture of dsRNAs for four of the RPTPs and varying all of the parameters that were likely to affect the efficacy of the technique. The dsRNAs used were from *Ptp10D*, *Dlar*, *Ptp69D* and *Ptp99A*. Because the RPTPs are homologous to each other, we designed the dsRNAs to have no perfect matches of >15 nucleotides to other sequences in the fly genome, in order to eliminate potential problems with cross-silencing. The parameters varied included: the age at which embryos were injected (i.e. precellularization versus postcellularization), injection volume, concentration of RNA delivered, placement of the drops within the embryo, and the amount of dessication the embryos were subjected to during the injection procedure. After injection, embryos were allowed to develop to stage 17 at 18°C. Embryos that formed midgut convolutions and wild-type head structures were scored as developmentally normal, and were dissected, fixed and stained with monoclonal antibodies (mAbs) 1D4 and 22C10, specific for axon tracts in the embryonic CNS.

We found that survival rates were excellent and embryonic development was not affected by injections of buffer alone (Table 1; Fig. 2d), with one caveat: injecting large volumes of buffer often caused embryos to rupture. We were concerned that age at time of injection might determine penetrance of the phenotype: perhaps cellularized embryos would be less affected by dsRNA because cell membranes had formed. We found that embryos injected after cellularization at stage 7 were more likely to rupture than younger embryos, and hence did not survive as well, but phenotypes that were assayed in embryos that did develop to stage 17 were as severe as those observed in embryos injected at earlier stages, consistent with RNAi results in other organisms [13].

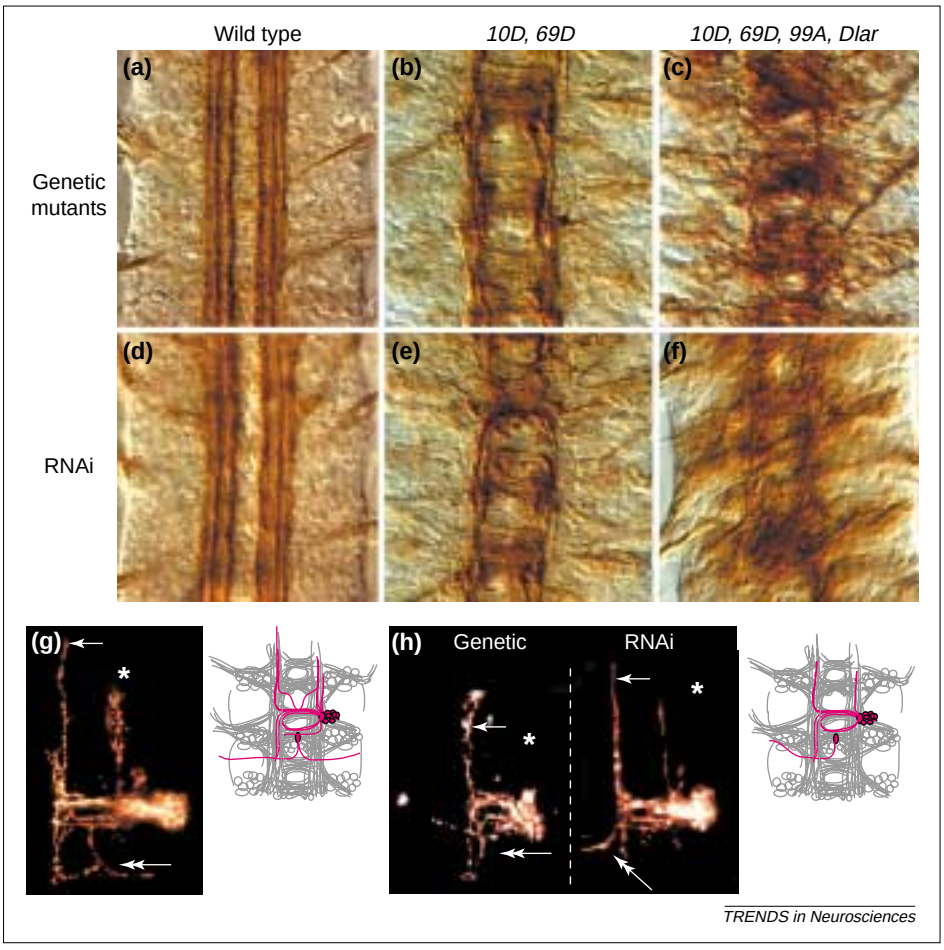

Fig. 2. RNAi phenocopies loss of function mutations. (a–f) Longitudinal axon pathways were observed using anti-Fasciclin II staining in wild-type, and in double- and quadruple- loss-of-function mutant embryos. These axonal patterns were compared with those seen in wild-type embryos injected with buffer or with equivalent dsRNAs and then similarly fixed and stained. (a) Wild-type stage-17 embryo. (b) Stage-17 embryo, homozygous mutant for *Ptp10D* and *Ptp69D*. (c) Stage-17 embryo, homozygous mutant for *Ptp10D*, *Ptp99A*, *Ptp69D* and *Dlar*. (d) Wild-type stage-17 embryo, injected with injection buffer only. (e) Wild-type stage-17 embryo, injected with dsRNA for *Ptp10D* and *Ptp69D*, each at 5 μM concentrations. (f) Wild-type stage-17 embryo, injected with dsRNA for *Ptp10D*, *Ptp99A*, *Ptp69D* and *Dlar*, each at 5 μM concentrations. See Ref. [9] for further details. (g) Wild-type NB 4-1 clone, labeled with DiI. The single white arrow points to long intersegmental interneuronal axons on the contralateral side, the asterisk marks ipsilateral intersegmental interneuronal projections and the double arrow points out the bifurcating motoneuron projecting into the transverse nerve (TN). See Ref. [10] for further details. The cartoon in (g) places the wt NB 4-1 clone in the context of the embryonic CNS. (h) *Ptp10D*, *Ptp69D* double-mutant NB 4-1 clones, whether genetically mutant (left) or generated using RNAi (right), display identical axonal defects. The ipsilateral interneuronal axons are severely diminished in number and extent of projection (asterisk). Contralateral intersegmental axons are less severely affected (single arrow). The TN motoneuron fails to bifurcate and sends only a single contralateral axon into the TN (double arrow). The cartoon in (h) places the *Ptp10D*, *Ptp69D* NB 4-1 clones in the context of the embryonic CNS.

Most importantly, we observed that injecting each dsRNA at concentrations of ≤2.5 μM produced variable phenotypes. Increasing the concentration to 5 μM for each dsRNA (20 μM total concentration, or about 6 mg ml^{-1} RNA) eliminated virtually all variability however, and we consistently observed phenotypes in mAb 1D4-stained embryos that were the same as those of quadruple null mutants (Table 1; Fig. 2; [9], note that dsRNA concentrations listed in the Methods section of this paper should read 20 μM where 1 μM is indicated).

Doubling the concentration again to 40 μM resulted in dsRNA crystallization within the injection needles. Because we are interested in investigating whether more than four genes can be silenced simultaneously (which would require total dsRNA concentrations of >20 μM under our current conditions), we are experimenting with changing injection buffer composition to increase dsRNA solubility, and using concentrations of each dsRNA that are >2.5 μM but < 5 μM.

We labeled NB lineages with DiI [10] following injection of multiple dsRNAs. An example of such a labeling experiment, for the NB 4-1 lineage, is shown in Fig. 2. The same alterations in DiI-labeled axon

Table 1. Parameters tested for injection of multiple dsRNAs in *Drosophila*[a]

Number of embryos injected	RNA concentration of each component (μM)	Stage at injection	Volume injected	Percentage survival to stage 17	Percentage of survivors showing null phenotype
22	0.0	5–6	small	86	0
10	0.0	5–6	medium	90	0
6	0.0	5–6	large	50	0
14	0.0	7+	small	72	0
16	0.0	7+	medium	88	0
7	0.0	7+	large	42	0
12	2.5	5–6	small	67	0
5	2.5	5–6	medium	100	~30
ND	2.5	5–6	large	ND	ND
10	2.5	7+	small	80	0
5	2.5	7+	medium	80	~30
ND	2.5	7+	large	ND	ND
12	5.0	5–6	small	67	100
11	5.0	5–6	medium	64	100
12	5.0	5–6	large	50	100
5	5.0	7+	small	80	100
6	5.0	7+	medium	50	100
6	5.0	7+	large	33	100

[a]Abbreviations: small, drop of ~25 μM radius; medium, drop of ~50 μM radius; large, drop of ~75 μM radius; ND, not determined.

pathways are produced by a double null mutation (for *Ptp10D* and *Ptp69D*) [8,9] and by injection of the two dsRNAs for these genes. This experiment opens the door to the possibility of inhibiting expression of two or more members of any protein family and observing directly how this inhibition affects any axon pathway in the embryonic CNS.

How many molecules of dsRNA per cell are required to achieve 100% phenotypic penetrance when four different RNAs are injected into *Drosophila* embryos? An injected spherical drop of dsRNA solution with a 50 μM radius (typical for embryo injection under standard conditions) that contains 5 μM of each dsRNA corresponds approximately to 1500 molecules of each dsRNA per embryonic cell. Our results suggest that there is a threshold for phenotypes that require several genes to be silenced by RNAi, because lowering the concentration of dsRNA (for the same size of injected drop) by only a factor of two produced a >50% decrease in penetrance of the severe quadruple mutant phenotype; and when smaller drops were used at a lower concentration, the phenotype was never observed (Table 1).

Two factors might help explain why such high concentrations of dsRNA are required to achieve high-penetrance silencing of multiple genes. First, the presence of multiple gene sequences in the injection mix can reduce the efficiency of inhibition for each individual gene. In a careful study of dsRNA inhibition of luciferase (Luc) gene expression from plasmid templates injected into *Drosophila* embryos [14], it was found that adding a large excess of unrelated dsRNA to the injection mix greatly reduced the efficiency of Luc gene silencing by low concentrations of dsRNA (0.075 μM). However, we note that at a 1:3 ratio of Luc dsRNA:unrelated dsRNA (comparable to the ratio of each dsRNA to the amount of the other dsRNAs when four genes are being perturbed), inhibition of Luc gene expression was only reduced by 50% (from 30-fold to 15-fold). This suggests that silencing four genes at once should not usually present major problems. Second, in earlier work on *Drosophila* [5], it was estimated that achieving 25% phenotypic penetrance for a single gene (*ftz*) required about 30 dsRNA molecules per cell (similar to the results for *C. elegans* [1]), but to reach >75% penetrance 100-fold dsRNA was required. Because it is crucial to achieve high-penetrance silencing of all the genes represented by dsRNAs when performing combinatorial RNAi experiments, our results indicate that the highest possible concentrations of dsRNA should be injected. Because such high concentrations have the potential to produce nonspecific toxicity (either through contaminants in the dsRNA or through an excess of injected nucleic acid), it is important to perform careful specificity controls when conducting these experiments.

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