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Gene Therapy of Human Severe Combined Immunodeficiency (SCID)–X1 Disease

Marina Cavazzana-Calvo,*[1,2,3] Salima Hacein-Bey,*[1,2,3] Geneviève de Saint Basile,[1] Fabian Gross,[2] Eric Yvon,[3] Patrick Nusbaum,[2] Françoise Selz,[1] Christophe Hue,[1,2] Stéphanie Certain,[1] Jean-Laurent Casanova,[1,4] Philippe Bousso,[5] Françoise Le Deist,[1] Alain Fischer[1,2,4]†

Severe combined immunodeficiency–X1 (SCID-X1) is an X-linked inherited disorder characterized by an early block in T and natural killer (NK) lymphocyte differentiation. This block is caused by mutations of the gene encoding the γc cytokine receptor subunit of interleukin-2, -4, -7, -9, and -15 receptors, which participates in the delivery of growth, survival, and differentiation signals to early lymphoid progenitors. After preclinical studies, a gene therapy trial for SCID-X1 was initiated, based on the use of complementary DNA containing a defective γc Moloney retrovirus–derived vector and ex vivo infection of $CD34^+$ cells. After a 10-month follow-up period, γc transgene–expressing T and NK cells were detected in two patients. T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls. Thus, gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit.

In considering diseases that might be ameliorated by gene therapy, a setting in which a selective advantage is conferred by transgene expression, in association with long-lived transduced cells such as T lymphocytes, may prove critical. SCID-X1 offers a reliable model for gene therapy because it is a lethal condition that is, in many cases, curable by allogeneic bone marrow transplantation (*1*–*4*). It is caused by γc cytokine receptor deficiency that leads to an early block in T and NK lymphocyte differentiation (*1*–*3*). In vitro experiments of γc gene transfer have shown that γc expression can be restored (*5*–*7*), as well as T and NK cell development (*8*–*9*), while the immunodeficiency of γc^- mice can be corrected by ex vivo γc gene transfer into hematopoietic precursor cells (*10*, *11*). Long-term expression of human γc has also been achieved by retroviral infection of canine bone marrow (*12*). It has been anticipated that γc gene transfer should confer a selective advantage to transduced lymphoid progenitor cells because, upon interaction with interleukin-7 (IL-7) and IL-15, the γc cytokine receptor subunit transmits survival and proliferative signals to T and NK lymphocyte progenitors, respectively (*2*, *3*). This hypothesis received further support from the observation that a spontaneously occurring γc gene reverse mutation in a T cell precursor in one patient led to a partial, but sustained, correction of the T cell deficiency, including at least 1000 distinct T cell clones (*13*, *14*). Spontaneous correction of the immunodeficiency has otherwise not been observed in several hundred γc-deficient SCID patients nor in γc^- mice (*2*–*4*).

Two patients, aged 11 months (P1) and 8 months (P2), with SCID-X1 met the eligibility criteria for an ex vivo γc gene therapy trial. SCID-X1 diagnosis was based on blood lymphocyte phenotype determination and findings of γc gene mutations resulting either in a tailless receptor expressed at the membrane (P1) (R289 X) or in a protein truncated from the transmembrane domain that was not expressed at cell surface (P2) (a frameshift causing deletion of exon 6) (*15*). After marrow harvesting and $CD34^+$ cell separation, 9.8×10^6 and 4.8×10^6 $CD34^+$ cells per kilogram of body weight from P1 and P2, respectively, were preactivated, then infected daily for 3 days with the MFG γc vector–containing supernatant (*16*). $CD34^+$ cells (19×10^6 and 17×10^6/kg, respectively) were infused without prior chemoablation into P1 and P2, ~20 to 40% and 36% of which expressed the γc transgene as shown by either semiquantitative PCR analysis (P1) or immunofluorescence (P2). As early as day +15 after infusion, cells carrying the γc transgene were detectable by PCR analysis (*17*) among peripheral blood mononuclear cells. The fraction of positive peripheral blood mononuclear cells increased with time (Fig. 1). T lymphocyte counts increased from day +30 in P1 (who had a low number of autologous T cells before therapy), whereas γc-expressing T cells became detectable in the blood of P2 at day +60 (Fig. 2). Subsequently, T cell counts, including $CD4^+$ and $CD8^+$ subsets, increased to 1700/μl from day +120 to +150 and reached values of ~2800/μl after 8 months (Fig. 2). Transgenic γc protein expression could not be studied on P1 cells given the presence of the endogenous tail-less protein. However, semiquantitative PCR performed at day +150 showed that a high proportion of T cells carry and express the γc transgene (Fig. 1, A and B). Similar results were observed at day +275. Southern blot analysis of provirus integration in peripheral T cells from both patients revealed a smear indicating that multiple T cell precursors had been infected by the retroviral vector (*18*).

Immunofluorescence studies showed that γc was expressed on the membrane of T cells in P2. The magnitude of expression was similar to that of control cells (Fig. 3A), as found in previous in vitro gene transfer experiments (*5*, *8*, *9*). These results indicate that sufficient transgene expression had been achieved and that γc membrane expression is likely to be regulated by the availability of the other cytokine receptor subunits with which γc associates (*3*). Both αβ and γδ T

[1]INSERM Unit 429, [2]Gene Therapy Laboratory, [3]Cell Therapy Laboratory, [4]Unité d'Immunologie et d'Hématologie Pédiatriques, Hôpital Necker, 75743 Paris Cedex 15, France. [5]INSERM Unit 277, Institut Pasteur, 75730 Paris, France.

*These authors contributed equally to this work.
†To whom correspondence should be addressed at INSERM Unit 429, Hôpital Necker–Enfants Malades, 149 rue de Sèvres, 75743 Paris Cedex 15, France. E-mail: fischer@necker.fr

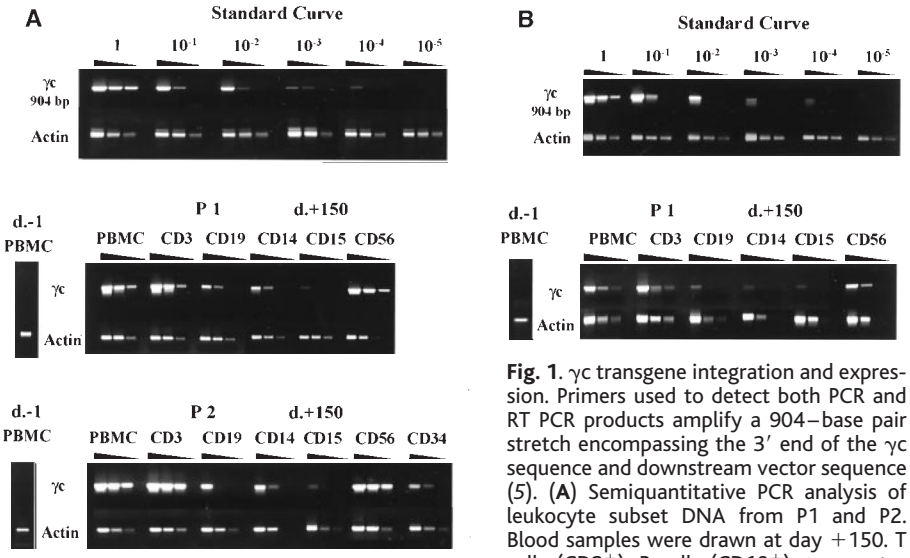


Fig. 1. γc transgene integration and expression. Primers used to detect both PCR and RT PCR products amplify a 904–base pair stretch encompassing the 3′ end of the γc sequence and downstream vector sequence (*5*). (**A**) Semiquantitative PCR analysis of leukocyte subset DNA from P1 and P2. Blood samples were drawn at day +150. T cells ($CD3^+$), B cells ($CD19^+$), monocytes ($CD14^+$), granulocytes ($CD15^+$), and NK cells ($CD56^+$) as well as $CD34^+$ from a bone marrow sample obtained at day +150 from P2 were isolated by a FACStar plus cell sorter (Becton Dickinson) after staining with appropriate mAbs (*19*). Purity was >99%. Sorted cells were analyzed for the frequency of vector-containing cells (*17*). Actin DNA was amplified in parallel. Samples from peripheral blood mononuclear cells (PBMC) obtained before treatment are shown as negative controls. A standard curve was constructed by diluting cells containing one copy of the MFG γc vector (*5*) with noninfected cells. All specimens were tested at three dilutions: 1:1, 1:20, and 1:200. (**B**) Semiquantitative RT-PCR analysis of leukocyte-subset RNA from P1. The same blood sample as in (A) was used. Actin cDNA was amplified in parallel as a control of RNA content. The standard curve was constructed as in (A) (*17*). No signal was detected in the absence of reverse transcriptase (not shown). Each specimen was diluted to 1:1, 1:500, and 1:5000.

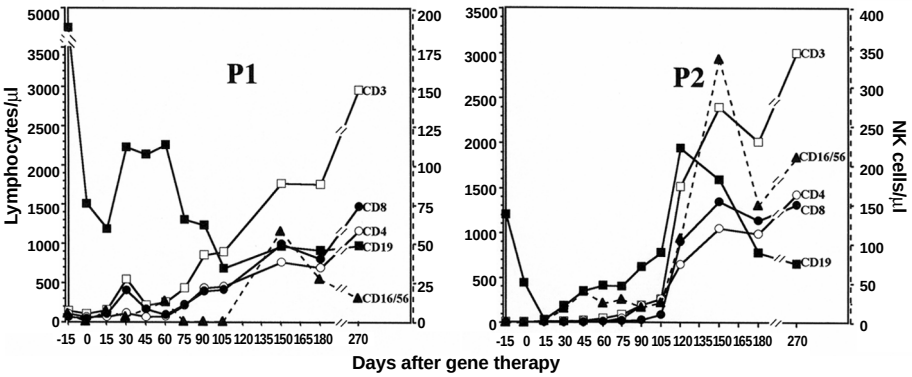
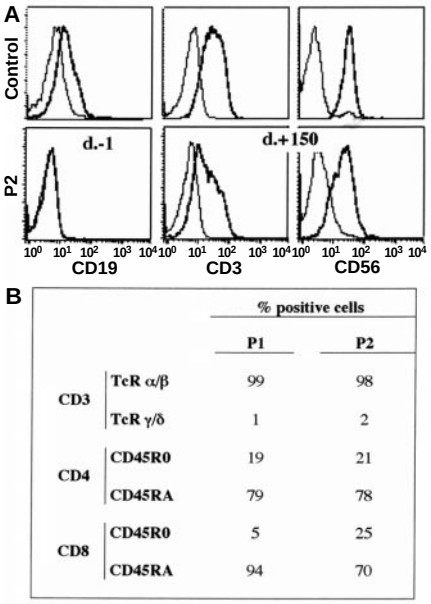


Fig. 2. Longitudinal study of lymphocyte subsets from patient 1 (P1) and patient 2 (P2). Absolute counts of T cells ($CD3^+$, $CD8^+$, and $CD4^+$), B cells ($CD19^+$), and NK cells ($CD16^+$, $CD56^+$) are shown as a function of time. Day 0 is the date of treatment. The scale for NK cells is on the right-hand side of each panel.



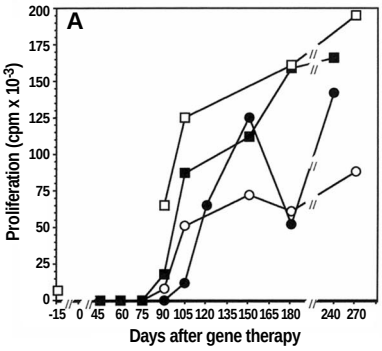
B

		% positive cells	
		P1	P2
CD3	TcR α/β	99	98
	TcR γ/δ	1	2
CD4	CD45R0	19	21
	CD45RA	79	78
CD8	CD45R0	5	25
	CD45RA	94	70

Fig. 3. γc protein expression and lymphocyte subsets. (**A**) γc protein detection at the surface of lymphocyte subsets from a control and from P2 obtained at day +150. γc expression on B cells from P2 after treatment was undetectable (not shown). The *y* axis depicts the relative cell number, and the *x* axis shows the logarithm of arbitrary immunofluorescence units. Thin lines are isotype controls; thick lines, staining by the anti-γc. Similar results were observed on blood samples obtained at days 275 (P1) and 240 (P2). (**B**) The percentage of $CD45RO^+$ and $CD45RA^+$ among CD4 and CD8 T cells from P1 and P2 obtained at day +275 and 240, respectively, as well as the percentage of T cells expressing either an αβ TCR or a γδ TCR.

cell receptor (TCR)–expressing T cells were detected (Fig. 3B). Polyclonality and V_β TCR diversity were demonstrated by using antibodies specific for TCR V_β (*19*) and the immunoscope method (*18*, *20*). In both patients, naïve $CD45RA^+$ T cells were detected, accounting for a majority of the T cell subset (Fig. 3B). In both patients, T cells proliferated from day +105 in the presence of phytohemagglutinin (PHA) and antibodies to CD3 (anti-CD3). The extent of proliferation was the same as that of age-matched controls (Fig. 4A). After primary vaccination, in vitro T cell proliferative responses to tetanus toxoid (P1 and P2: 18,000 and 12,000 cpm, respectively) and polioviruses (P2: 38,000 cpm) were observed within normal range (*21*). P1 T cells were also found to proliferate in the presence of protein pure derivitive (PPD) (12,000 cpm) as a likely consequence of bacillus Calmette-Guerin (BCG) persistence after immunization at 2 months of age in this immunocompromised child. Five months after cessation of intravenous immunoglobulin (Ig) therapy, antibodies to tetanus and diphtheria toxoids as well as to polioviruses were found in the serum of both patients, together with detectable concentrations of IgG and IgM (Fig. 4B). A normal level of IgA was also detected in the serum of P1. As determined by semi-quantitative PCR and reverse transcriptase–PCR analysis, it was observed that in both cases, a low fraction of B cells carry and express the γc transgene (Fig. 1). It is therefore unknown whether antibody responses are provided by untransduced or the few transduced B cells. Residual persistence (< 1%) of administered intravenous immunoglobulins (last given 5 months before measurement of antibody response) could, in part, also contribute. The γc-expressing NK cells were detected in the blood of P2 by day 30 (Figs. 1, 2, and 3A). These cells efficiently killed K562 cells in vitro (*18*). NK cells became detectable in the blood of P1 only from day +150.

As a likely consequence of development and sustained function of the immune system, clinical improvement was observed in both patients. In P2, protracted diarrhea as well as extensive graft-versus-host disease (GVHD)–like skin lesions disappeared. Both patients left protective isolation at days 90 and 95 and are now at home 11 and 10 months, respectively, after gene transfer without any treatment. Both enjoy normal growth and psychomotor development. No side effects have been noted. A similar result has since been achieved in a third patient 4 months



B

	IgG	IgA	IgM	Antibodies to : Tet. tox. (iu/ml)	Dipht. Tox. (iu/ml)	Polio virus I II III Titer x 10^{-1}
	mg/dl					
P1 (d. + 270)	323	41	202	.53	.86	320 160 160
P2 (d. + 240)	309	0	46	.93	.63	640 640 160
Control (age matched)	420-850	16-80	40-90	>.20	>.20	>80

Fig. 4. Functional characteristics of transduced lymphocyte subsets. (**A**) Longitudinal follow-up of PHA (□, ■)– and anti-CD3 (○, ●)–induced proliferation of lymphocytes from P1 (open symbols) and P2 (filled symbols) (*8*). Background [^{3}H]thymidine uptake was less than 400 cpm. Positive control values are $>50 \times 10^3$ cpm. (**B**) Serum immunoglobulin analysis was determined by nephelometry and serum antibody by enzyme-linked immunosorbent assay after immunization (see above). Diphtheria toxoid (Dipht. tox.) was also used for immunization. The last intravenous Ig injections were given at day +90 in both patients. Tet. Tox., tetanus toxoid. Isohemagglutinins to blood group A have now been detected in both patients' sera.

after gene transfer (*22*). These results demonstrate that in these patients, a selective advantage was conferred to T and NK lymphocyte progenitors, enabling full-blown development of mature and functioning T and NK lymphocytes (*23*).

These overall positive results contrast with the failure of previous attempts to perform ex vivo gene therapy in adenosine deaminase (ADA)–deficient patients (*24–27*). Concomitant administration of ADA enzyme to these patients is likely to have counterbalanced the potential growth advantage of the transduced cells in this setting (*23*). Also, advances in the methodology of gene transfer into CD34$^+$ cells, i.e., the use of a fibronectin fragment (*28*) as well as of a cytokine combination enabling potent CD34 cell proliferation, contributed to the success of γc gene therapy.

Because γc gene transfer was achieved without any additional myeloablative or immunosuppressive therapy, these results pave the way for a possible extension of this therapeutic approach to other genetic diseases characterized by defective cell-subset generation, such as other forms of SCID (*29*). The kinetics of T cell development in γc gene transfer is similar to that observed in SCID patient recipients of haploidentical stem cell transplantation (*4*), suggesting that early progenitor cells have been infected by the MFG γc virus and effectively transduced. The hypothesis that transduced autologous T cells in P1 account for the development of the T cell compartment is unlikely because (i) the infected CD34$^+$ cell population was contaminated by less than 0.1% CD3$^+$ T cells; (ii) a thymic gland (27 mm by 25 mm by 25 mm at day +275) became detectable by ultrasound echography, indicative of thymopoiesis, whereas most T cells at day +275 exhibit a naïve CD45RA$^+$ phenotype; and (iii) the T cell repertoire was polyclonal and diverse. In both patients, it was shown that at day +150, a fraction of bone marrow CD34$^+$ cells harbored and expressed the γc transgene (Fig. 1, P2). It was not possible to determine whether more primitive cells, i.e., CD34$^+$CD38$^-$ cells, were transduced because of insufficient bone marrow sample. In the mouse, a common lymphoid progenitor (CLP) gives rise to the different lymphocyte populations (*30*). If a human counterpart of CLP exists, it would be the best candidate from among the earliest cells that were transduced ex vivo from these patients. Identification of integration sites in the various cell lineages could help determine the permissive differentiation stage. The question of the persistence of T and NK cell generation has yet to be addressed. If infected cells have no self-renewal capacity and have a short life-span, new generation of T and NK cells should cease. However, the fact that a thymic gland is still detectable 9 months after γc gene transfer suggests that thymopoiesis is still ongoing. Follow-up of the SCID-X1 patient in whom a spontaneous reversion mutation occurred in a T cell precursor (*13*, *14*) indicates that gene transfer could be sufficient to provide a functional memory T cell pool for a number of years. This optimistic view will require careful sequential appraisal. Kohn *et al.* have previously shown that transgenes placed under the control of the long-terminal repeat (LTR) viral promoter can be silenced in quiescent T cells (*31*). Although the identification of silencing sequences in the MFG LTR makes this a strong possibility (*31*), down-regulation of γc expression has not been observed so far in these two patients, in γc-deficient mice treated by ex vivo γc gene transfer (*11*), or in cell lines maintained in culture over 1 year (*5*).

Follow-up will be required to assess the long-term effects of ex vivo γc gene transfer in CD34$^+$ cells of SCID-X1 patients. To date, this methodology has resulted in the sustained correction (up to 10 months) of the SCID-X1 phenotype in two patients, including a patient in whom the mutated protein is expressed at the cell surface. It is presumed that the effect results from a strong positive selective pressure provided to the corrected lymphoid progenitors.

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15. Patient 1 had pneumocystis carinii pneumonitis and had received BCG immunization. Patient 2 suffered from recurrent oral candidiasis, pneumocystis carinii infection, protracted diarrhea, failure to thrive, and GVHD-like skin lesions. Neither patient had an HLA (human leukocyte antigen)–identical sibling. Patients were placed in a sterile isolation ward and received nonabsorbable oral antibiotics and intravenous Igs every 3 weeks for 3 months. Parents gave informed consent for participation in the trial.
16. The defective MFG γc vector has been described previously (*5*). It was packaged in the ψcrip cell line. The MFG γc vector–containing supernatant was manufactured and provided by Genopoietic (Lyon, France) under GMP guidelines. The vector supernatant was free of replication-competent retrovirus as determined by S+L- assay and a β-galactosidase mobilization test [R. H. Bassin, N. Tuttle, P. J. Fischinger, *J. Cancer* **6**, 95 (1970); M. Printz *et al.*, *Gene Ther.* **2**, 143 (1995)]. Concentration of the virus in the supernatant was 5×10^5 infectious virus particles (*5*). Marrow CD34$^+$ cells were positively selected by an immunomagnetic procedure (CliniMACS, Miltenyi Biotec, Bergish Gladbach, Germany). CD34 cells were cultured in gas-permeable stem cell culture (PL-2417) containers (Nexell Therapeutics, Irvine, CA), at a concentration of 0.5×10^6 cells/ml in X-vivo 10 medium (Biowhittaker, Walkerville, MD) containing 4% fetal cell serum (Stem Cell Technologies, Vancouver, Canada), stem cell factor (300 ng/ml, Amgen), polyethylene glycol–megabaryocyte differentiation factor (100 ng/ml, Amgen), IL-3 (60 ng/ml, Novartis), and Flt3-L (300 ng/ml, R&D Systems, Minneapolis, MN) for 24 hours at 37°C in 5% CO_2. Containers were precoated with the CH296 human fragment of fibronectin (50 μg/ml) (TaKaRa, Shiga, Japan). Retroviral containing supernatant was added every day for 3 days. Cells were then harvested, washed twice, and infused back into the patients.
17. For semiquantitative PCR and RT-PCR analysis, DNA was isolated from the indicated cell populations. A reference standard curve was constructed by diluting cells from a SCID-X1–derived Epstein-Barr virus (EBV)–B cell line containing one copy per cell of the MFG γc provirus (*5*) in uninfected cells from the same EBV-B cell line (100, 10, 1, 0.1, 0.01, and 0.001%). DNA from each sample was also quantified by actin gel amplification. MFG γc primers sequences and

actin primers sequences are available on request. DNA was amplified in a 50 μl of PCR reaction mixture by using 30 cycles at an annealing temperature of 60°, for γc primers and 68°C for actin primers. A sample of the amplified product was separated on a 1% agarose gel and analyzed by ethidium bromide staining. RNA was prepared with the RNA easy kit (Qiagen) and was reverse-transcribed with the Superscript Preamplification System (Gibco-BRL). γc proviral and β-actin cDNA amplification were performed as described above. Quantification of expression was made by comparison with RNA isolated from the same standard curve of diluted cells.

18. M. Cavazzana-Calvo *et al*., data not shown.
19. The following monoclonal antibodies (mAbs) were used in immunofluorescence studies: anti-γc chain: Tugh 4 (rat IgG2, PharMingen, San Diego, CA); anti-CD3: Leu 4 (IgG2a, Becton Dickinson, San Diego, CA); anti-CD4: Leu3a (IgG1, Becton Dickinson); anti-CD8: Leu 2a (IgG1, Becton Dickinson); anti-CD19: J4 119 (IgG1 Immunotech, Marseille, France); anti-CD14: Leu M3 (Becton Dickinson); anti-CD16: 3G8 (IgG1, Immunotech); anti-CD56: MY31 (IgG1, Becton Dickinson); anti-CD15 (IgM, PharMingen); anti-TCR αβ: BMA031 (IgG1, Immunotech); anti-TCR γδ: IMMU 515 (IgG1, Immunotech); anti-CD45RO: UCHL1 (IgG2a, Immunotech); anti-CD45RA: 2H4 (IgG1, Coulter Clone, Margency, France); anti-CD34: HPCA-2 (IgG1, Becton Dickinson); anti-TcR Vβ2: MPB2D5 (IgG1, Immunotech); anti-TcR Vβ3: CH92 (IgM, Immunotech); anti-TcR Vβ5.1: IMMU 157 (IgG2a, Immunotech); anti-TcR Vβ5.2: 36213 (IgG1, Immunotech); anti-TcR Vβ5.3: 3D11 (IgG1, Immunotech); anti-TcR Vβ8: 56C5.2 (IgG2a, Immunotech); anti-TcR Vβ9: FIN9 (IgG2a, Immunotech); anti-TcR Vβ13.1: IMMU 222 (IgG2, Immunotech); anti-TcR Vβ13.6: JU74.3 (IgG1, Immunotech); anti-TcR Vβ14: CAS1.13 (IgG1, Immunotech); anti-TcR Vβ17: E17.5F3.15.13 (IgG1, Immunotech); anti-TcR Vβ21.3: IG125 (IgG2, Immunotech). Fluorescence staining was done with phycoerythrin- or fluorescein isothiocyanate–conjugated mAbs. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson).
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22. This patient was treated at 1 month of age. Within 3 months, T and NK lymphocyte counts reached age-matched control values. The γc expression at T and NK cell surfaces was fully restored. The child is at home without any therapy, 4 months after treatment.
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Localization of a Short-Term Memory in *Drosophila*

T. Zars,[1]*† M. Fischer,[1]* R. Schulz,[2] M. Heisenberg[1]

Memories are thought to be due to lasting synaptic modifications in the brain. The search for memory traces has relied predominantly on determining regions that are necessary for the process. However, a more informative approach is to define the smallest sufficient set of brain structures. The *rutabaga* adenylyl cyclase, an enzyme that is ubiquitously expressed in the *Drosophila* brain and that mediates synaptic plasticity, is needed exclusively in the Kenyon cells of the mushroom bodies for a component of olfactory short-term memory. This demonstrates that synaptic plasticity in a small brain region can be sufficient for memory formation.

The localization of memory traces has occupied neuroscientists throughout this century (*1*). Approaches have ranged from surgical ablation to mapping localized necessary gene expression in transgenic animals (*2*, *3*). Until recently, attempts to localize a memory trace have relied mainly on determining necessary brain regions (*4*). However, in a highly integrated network, other components besides the one being studied may also be necessary.

In insects, much attention has been paid to the mushroom bodies as the site for olfactory learning (*3*, *5*–*8*). In *Drosophila*, they are made up of about 2500 intrinsic neurons (Kenyon cells), receive multimodal sensory input, preferentially from the antennal lobe to the calyx, and send axon projections to the anterior brain where they bifurcate to form the α/β, α′/β′, and γ lobes (*9*). Noninvasive intervention techniques can provide mushroom body–less flies. In most respects, these flies show remarkably normal behavior but are deficient in olfactory learning (*5*). Genes important for olfactory memory have elevated expression levels in the mushroom bodies (*6*, *8*). Additionally, the mushroom bodies are necessary for context generalization in visual learning at the flight simulator and the control of spontaneous walking activity (*10*, *11*).

The *rutabaga* (*rut*) gene of *Drosophila* encodes a type I Ca^{2+}/calmodulin-dependent adenylyl cyclase (AC). Regulated synthesis of cyclic adenosine 3′,5′-monophosphate by

[1]Theodor Boveri Institut für Biowissenschaften, Lehrstuhl für Genetik, (Biozentrum) Am Hubland, D97074, Würzburg, Germany. [2]Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: zars@biozentrum.uni-wuerzburg.de

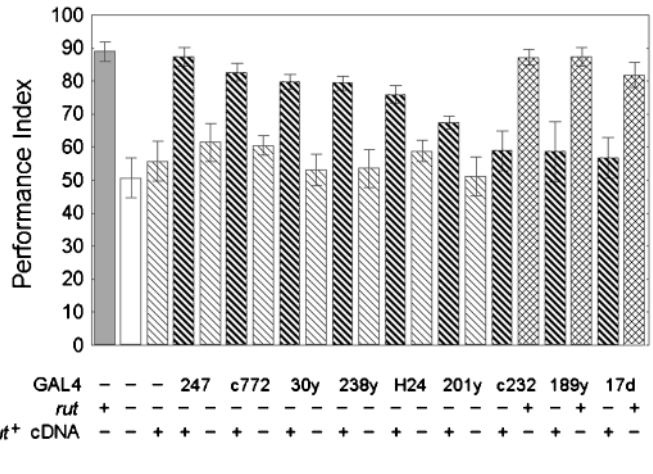

Fig. 1. The *rut* mutant defect in olfactory short-term memory can be rescued with a rut^+ cDNA in several GAL4 enhancer trap lines. Memory was measured about 2 min after classical conditioning (*17*). Performance indices (PIs) of *rut* mutant flies (white bar) and *rut* mutant flies with either a P[UAS_{GAL4}-rut^+] or GAL4 enhancer trap element (thin diagonal striped bars) were significantly different from wild-type flies (dark gray bar; P's < 0.0005). There was no significant difference between *rut* mutant flies' PIs rescued with GAL4 enhancer trap elements 247, c772, 30y, 238y, and H24 and the P[UAS_{GAL4}-rut^+] compared with wild-type flies (dark gray and thick diagonal striped bars, respectively; P's > 0.05). Mutant *rut* flies' performance was rescued with GAL4 element 201y and the P[UAS_{GAL4}-rut^+] (P < 0.05) but was also significantly lower than the performance of wild-type flies (P < 0.005). GAL4 enhancer trap lines c232, 189y, and 17d with a P[UAS_{GAL4}-rut^+] did not rescue the *rut* mutation (P's > 0.05). Wild-type flies heterozygous for GAL4 enhancer trap elements c232, 189y, and 17d were not significantly different from wild-type flies (dark gray and cross-hatched bars; P > 0.05). Bars represent mean PIs; errors are SEMs; n = 6 for all genotypes.