

## Rapid Report

### Recombinant GABA<sub>C</sub> receptors expressed in rat hippocampal neurons after infection with an adenovirus containing the human $\rho 1$ subunit

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1. A recombinant adenovirus was generated with the human  $\rho 1$  GABA<sub>C</sub> receptor subunit (adeno- $\rho$ ). Patch-clamp and antibody staining were employed to confirm functional expression of recombinant  $\rho 1$  receptors after infection of human embryonic kidney cells (HEK293 cell line), human embryonic retinal cells (911 cell line), dissociated rat hippocampal neurons and cultured rat hippocampal slices.
2. Standard whole-cell recording and Western blot analysis using  $\rho 1$  GABA<sub>C</sub> receptor antibodies revealed that recombinant  $\rho 1$  receptors were expressed in HEK293 and 911 cells after adeno- $\rho$  infection and exhibited properties similar to those of  $\rho 1$  receptors after standard transfection.
3. Cultured rat hippocampal neurons (postnatal day (P)3–P5) did not show a native GABA<sub>C</sub>-like current. After adeno- $\rho$  infection, however, a GABA<sub>C</sub>-like current appeared in 70–90% of the neurons.
4. Five days after infection, expression of GABA<sub>C</sub> receptors in hippocampal neurons significantly decreased native GABA<sub>A</sub> receptor currents from  $1200 \pm 300$  to  $150 \pm 70$  pA ( $n = 10$ ). The native glutamate-activated current was unchanged.
5. Hippocampal slices (P8) did not show a native GABA<sub>C</sub>-like current, although recombinant  $\rho 1$  receptors could be expressed in cultured hippocampal slices after adeno- $\rho$  infection.
6. These data indicate that an adenovirus can be used to express recombinant GABA<sub>C</sub> receptors in hippocampal neurons. This finding could represent an important step towards the gene therapy of CNS receptor-related diseases.

The ligand-gated chloride channels including  $\rho$  GABA<sub>C</sub> and  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\pi$ ,  $\delta$  and  $\theta$  GABA<sub>A</sub> receptors are key elements in the tonic and synaptic inhibitory signalling in the CNS (Cutting *et al.* 1991; MacDonald & Olsen, 1994; Wang *et al.* 1994). Unlike GABA<sub>C</sub> receptors, GABA<sub>A</sub> receptors are reversibly blocked by bicuculline and modulated by barbiturates and benzodiazepines (Polenzani *et al.* 1991; Shimada *et al.* 1992). GABA<sub>A</sub> receptors are widely distributed in the retina, spinal cord, hippocampus, cerebellum, superior colliculus, thalamus and other brain regions (Houser *et al.* 1988; Zimprich *et al.* 1991; Feigenspan & Bormann, 1994; MacDonald & Olsen, 1994). GABA<sub>C</sub> receptors are widely expressed in the retina, with lower levels in the brain and spinal cord (Strata & Cherubini, 1994; Zhang *et al.* 1995; Enz *et al.* 1996; Koulen *et al.* 1998; Lukasiewicz & Shields, 1998).

Numerous CNS diseases such as epilepsy, hepatic encephalopathy, spinocerebellar degeneration and dementia may be associated with a functional abnormality of GABAergic transmission (Cossart *et al.* 2001). A potential method to treat these abnormalities is the delivery of the DNA coding for functional GABA receptors into the disease-affected tissue. The human adenovirus (serotypes 2, 5) is a potentially powerful gene-delivery vehicle in that it satisfies the following stringent criteria: (i) high level of transduction, (ii) high insert capacity, (iii) wide variety of cell targets, (iv) amplification to very high titres, (v) non-oncogenic, and (vi) replication deficient (Douglas & Curiel, 1997; Krasnykh *et al.* 2000). The prime receptor for the human adenovirus (serotypes 2, 5) is shown to be similar to that for coxsackie B virus and has therefore been termed the coxsackie/adenovirus receptor

(CAR) (Roelvink *et al.* 1998). Biochemical analysis of CAR revealed that it is a 46 kDa glycoprotein widely distributed in human fibroblasts, glia, and to a lesser extent in the differentiated respiratory epithelium, mature skeletal muscle and human lymphocytes (Zabner *et al.* 1997; Walters *et al.* 1999; Nalbantoglu *et al.* 1999; Hidaka *et al.* 1999). Less is known about CAR distribution in neuronal cells.

In this study we have used adenovirus serotype 5 to deliver DNA encoding the  $\rho 1$  GABA<sub>C</sub> receptor subunit into neuronal hippocampal cells. Recombinant adenovirus containing the  $\rho 1$  subunit (adeno- $\rho$ ) was produced under the human cytomegalovirus (CMV) promoter. Recombinant  $\rho 1$  GABA<sub>C</sub> receptors were expressed in 70–90% of cultured hippocampal neurons after adeno- $\rho$  infection. Patch-clamp analysis of GABA-activated current revealed that the  $\rho 1$  receptors had similar properties to  $\rho 1$  receptors expressed using standard transfection methods in non-neuronal cells. This finding could represent an important step towards the gene therapy of CNS receptor-related diseases.

## METHODS

### Molecular biology

The human  $\rho 1$  subunit and rat  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$  subunits were obtained from cDNA libraries via the polymerase chain reaction as described previously (Amin *et al.* 1994; Amin & Weiss, 1994). The cDNA of the  $\rho 1$  subunit was excised using *Bam*HI and *Xba*I restriction enzymes and inserted in the pShuttle CMV vector using *Bgl*II and *Xba*I ligation sites. Recombinant adenovirus containing  $\rho 1$  under the control of the human CMV promoter was produced using the QuantumAdEasy kit (Quantum Biotechnologies, Quebec, Canada) and has been termed adeno- $\rho$ . Adeno- $\rho$  was propagated in 10<sup>9</sup> HEK293 cells and was purified by centrifugation in a CsCl gradient according to Quantum protocols. The titre of infectious viral particles of adeno- $\rho$  determined by plaque assay after large-scale purification was  $2 \times 10^{11}$  plaque-forming units (PFU) ml<sup>-1</sup>. Dialysed adeno- $\rho$  was aliquoted and stored at -80°C.

**Transfection.** HEK293 cells were transfected with  $\rho 1$  and/or  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$  subunits in the pCDNA3 vector using Fugene 6 (Roche, Indianapolis, IN, USA) as described by the manufacturer.  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$  were cotransfected at a cDNA ratio of 1:1:2 with a total of 4  $\mu$ g of cDNA per 35 mm dish. For the case of the cotransfection of  $\rho 1$ ,  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$ , the cDNA ratio was 1:1:1:2 with a total of 5  $\mu$ g of cDNA per 35 mm dish. In all cases, 1  $\mu$ g of green fluorescent protein (GFP) was included for visualization of transfected cells.

### Primary culture of hippocampal neurons and cell infection

For preparation of dissociated neurons, Sprague-Dawley rats at stage P3–P5 (Harlan, Indianapolis, IN, USA) were rapidly decapitated after cervical dislocation, and the hippocampi were removed from the brain and dissected free of meninges in cooled (6°C), oxygenated, phosphate-buffered saline (PBS) containing Ca<sup>2+</sup> and Mg<sup>2+</sup>. This procedure, as well as the procedure for obtaining hippocampal slices (described below), were carried out under the guidelines and approval of the UAB Institutional Animal Care and Use Committee. The hippocampi were then transferred into Ca<sup>2+</sup>, Mg<sup>2+</sup>-free PBS, cut into small pieces and incubated with 0.3% (w/v) protease from *Aspergillus oryzae* (Type XXIII; Sigma, St Louis, MO, USA) and 0.1% (w/v) DNase (Type I, Sigma) for 20 min at 25°C. The tissue was washed

and triturated. After a brief centrifugation, the cell pellet was resuspended in culture medium (minimal essential medium (MEM), Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% NU serum (Fischer Scientific, Pittsburgh, PA, USA), penicillin (5 U ml<sup>-1</sup>) and streptomycin (5  $\mu$ g ml<sup>-1</sup>), and plated at a density of 8–10 ( $\times 10^4$ ) cells cm<sup>-2</sup> on glass coverslips coated with poly-L-lysine. Cells were used after 10–14 days in culture.

Adeno- $\rho$  was used at a concentration of 100 PFU cell<sup>-1</sup> for the neuronal cultures, and in the range of 2 to 100 PFU cell<sup>-1</sup> for the HEK293 and 911 cell lines. GABA-activated currents were recorded from 12 h to 5 days after infection.

### Organotypic hippocampus slice culture and slice infection

Stage P7 Sprague-Dawley rats (Harlan) were cervically dislocated and rapidly decapitated. Hippocampal slices (200–400  $\mu$ m thick) were prepared with a custom-designed wire slicer and maintained *in vitro* on Millicell-CM filter inserts (Millipore, Bedford, MA, USA) in a 36°C, 5% CO<sub>2</sub>, humidified (99%) incubator (Stoppini *et al.* 1991). The concentration of horse serum (Gibco) in the culture medium was reduced from 20 to 10% at 6 days *in vitro*. Over the next 2 days, serum was reduced to 5% and then 0%. The culture medium was completely exchanged every 3 days. The best infection of slices was observed in serum-free medium.

### Electrophysiology

Experiments were performed at room temperature (20–24°C) using the whole-cell recording patch-clamp technique as previously described (Filippova *et al.* 1999). The holding potential was -50 mV. The external recording solution contained (mM): NaCl, 160; KCl, 3.5; glucose, 10; CaCl<sub>2</sub>, 2; and Hepes, 10 (pH 7.4). In some experiments TTX (1  $\mu$ M), DL-2-amino-5-phosphonopentanoic acid (DL-APV, 10  $\mu$ M), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10  $\mu$ M) or bicuculline (30–50  $\mu$ M) was added to the external bath solution to decrease spontaneous synaptic activity. The recording pipettes (borosilicate glass) had resistances of 3–5 M $\Omega$  when filled with internal solution containing (mM): CsCl, 150; CaCl<sub>2</sub>, 0.25; EGTA, 1.1 (free Ca<sup>2+</sup>,  $5 \times 10^{-8}$  M); Hepes, 10; and Mg-ATP, 4 (pH 7.2). GABA, glycine and glutamate were applied to the cells through a double-barrelled perfusion system. In some experiments, bicuculline (30–50  $\mu$ M) was added in the GABA-containing solution. In order to determine the GABA<sub>A</sub> and GABA<sub>C</sub> current amplitudes in native neurons infected with adeno- $\rho$ , the amplitude of the GABA<sub>A</sub> current was calculated by subtraction of the GABA<sub>C</sub> current amplitude (activated by 20  $\mu$ M GABA in the presence of 50  $\mu$ M bicuculline) from the current activated by 300  $\mu$ M GABA without bicuculline. In the case of the  $\rho 1$  and  $\rho 1$ - $\alpha 1\beta 2\gamma 2$  coexpression studies in HEK293 cells, the amplitude of the GABA<sub>A</sub> current was calculated by subtraction of the GABA<sub>C</sub> current amplitude (activated by 10  $\mu$ M GABA in the presence of 20  $\mu$ M bicuculline) from the current activated by 200  $\mu$ M GABA without bicuculline.

Dose–response relationships were fitted with the following Hill equation using a non-linear least-squares method:

$$I = I_{\max}/(1 + (EC_{50}/[A])^{n_H}),$$

where  $I$  is the peak current response at a given concentration of agonist ( $A$ ),  $I_{\max}$  is the maximum current response,  $EC_{50}$  is the concentration of agonist with half-maximal activation, and  $n_H$  is the Hill coefficient. Data were compared statistically by Student's  $t$  test. Statistical significance was determined at the 5% level. All results are presented as means  $\pm$  S.E.M.

### N-terminal GABA<sub>C</sub> receptor antibodies

N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibodies were raised against the GABA<sub>C</sub> receptor  $\rho 1$  subunit by synthesizing a fusion protein

corresponding to the  $\rho 1$  N-terminal region (positions 14–191) with a 6His tag on the N-terminus. The specific oligonucleotide primers used for the N-terminal fusion protein were as follows.

Forward primer (position 42–67): 5'-CCACGCGGATCCGGCCAC TGAAAGCAGAATGCACTGG-3'

Reverse primer (position 573–538): 5'-GACTGAGCCCAAGCTTCTA CATTGCAGTTACTGTAAACCTGAGACTATAGAGCAC-3'

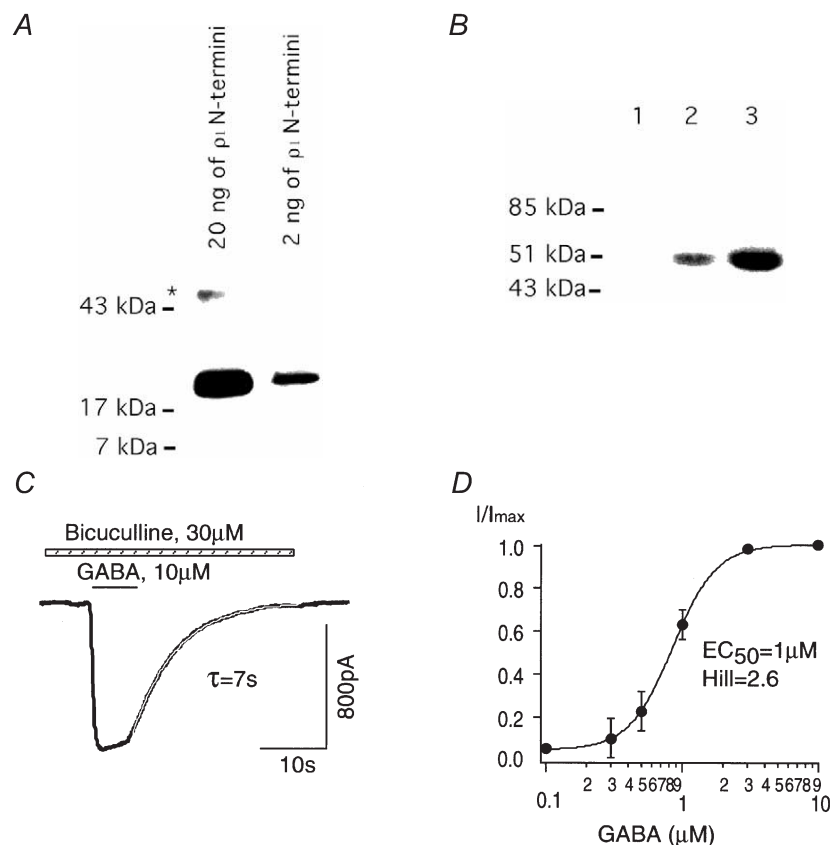
The PCR product was subcloned, using *Bam*HI and *Hind*III sites added to the primers, into the bacterial expression vector pQE-30 (Qiagen Inc., Valencia, CA, USA). The 6His fusion protein was expressed in *E. coli* BL21-Gold(DE3) pLysS cells (Stratagene, La Jolla, CA, USA) and was purified from urea-solubilized inclusion bodies by Ni-NTA chromatography (Qiagen) and then refolded. Mice were injected with the antigen and the serum was purified using an antigen-coupled, cyanogen bromide-activated column (Sigma). The mice were humanely killed at the end of the procedure, which was carried out under the guidelines and approval of the UAB Institutional Animal Care and Use Committee.

### Gel electrophoresis and Western blot analysis

HEK293 cells expressing  $\rho 1$  were lysed in cell culture lysis reagent (Promega, Madison, WI, USA). The concentration of total cell protein was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Total cell protein from HEK293 cells and bacterial fusion proteins were separated by SDS-PAGE, transferred to Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and detected using the ECL+Plus Western blotting detection system (Amersham Pharmacia Biotech). The following dilutions of antibody were used: anti-N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibody, 1:500 for HEK293 cell lysates and 1:2000 for *E. coli* fusion proteins; secondary sheep anti-mouse Ig horseradish peroxidase-linked antibody (Amersham Life Sciences), 1:2000.

### Figure 1. Properties of recombinant $\rho 1$ receptors expressed in HEK293 cells after adeno- $\rho$ infection

*A*, Western blot with N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibodies, which recognized a bacterially synthesized N-terminal fusion protein. The band indicated by the asterisk probably represents non-specific staining of an unidentified protein. *B*, Western blot from uninfected HEK293 cells (lane 1) and HEK293 cells infected with adeno- $\rho$  (2 and 10 PFU cell<sup>-1</sup>, lanes 2 and 3, respectively). Note the specific band of the  $\rho 1$  subunit of the GABA<sub>C</sub> receptor (50 kDa) in lanes 2 and 3. *C*, whole-cell current evoked at a holding potential of -50 mV with GABA (10  $\mu$ M) in the presence of bicuculline (30  $\mu$ M) from HEK293 cells after adeno- $\rho$  infection. Decay of the current upon GABA removal was well described by a single exponential component with a time constant ( $\tau$ ) of 7 s. *D*, mean dose-response relationship for GABA-activated current fitted with a Hill equation ( $n = 4$ ).



### Immunocytochemistry and laser scanning confocal microscopy

Slices or primary hippocampal cells were fixed overnight in 4 and 2% paraformaldehyde in PBS solution, respectively, rinsed in PBS, incubated in blocking solution (10% horse serum, 2% bovine serum albumin in PBS), and then incubated overnight at 4°C in primary antibody (anti-N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibody, 1:200). Following washout of the primary antibody with PBS solution, slices or cells were incubated overnight with the secondary antibody (Texas Red-conjugated antibody, Amersham Life Sciences) and mounted. Imaging was performed with a laser scanning confocal microscope (LSCM; Olympus Fluoview, Mellville, NY, USA). In some cases, to aid cell visualization, the membrane-permeable red fluorescent dye Ro31-8222 (Roche Molecular Biochemicals) was added to the external solution. Appropriate controls lacking primary and secondary antibodies were performed, and background fluorescence was adjusted for each experiment.

### Drugs

The following drugs were used for the experiments: bicuculline, GABA, glutamate, glycine (all from Sigma), CNQX, DL-APV5, 3-aminopropylphosphonic acid (3-APA), (2S)(+)-5,5-dimethyl-2-morpholineacetic acid (SCH 50911) and trans-4-aminocrotonic acid (TACA) (all from Tocris, Ballwin, MO, USA).

## RESULTS

### Characterization of recombinant $\rho 1$ receptors in HEK293 cells after adeno- $\rho$ infection

HEK293 cells were infected with adeno- $\rho$  at a concentration of 2–10 PFU cell<sup>-1</sup>. To confirm expression of recombinant  $\rho 1$  receptors, N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibodies were used in a Western blot analysis

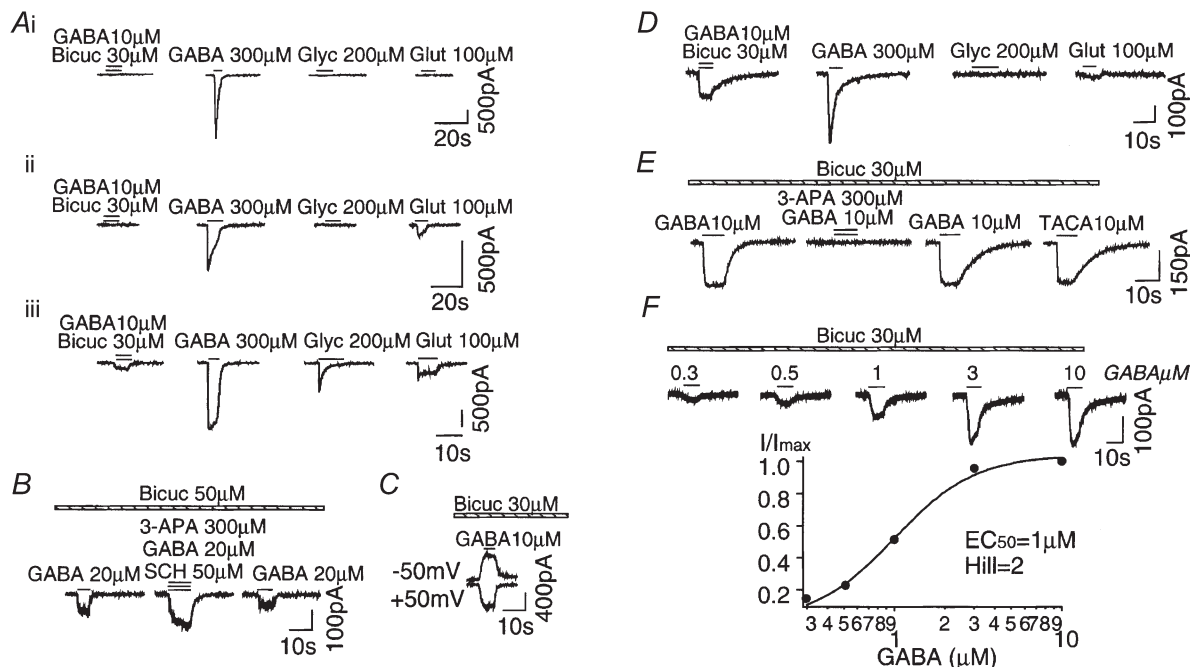
performed on HEK293 cells. Figure 1*A* demonstrates that the N-terminal  $\rho 1$  antibody recognized a bacterially synthesized N-terminal fusion protein of the human  $\rho 1$  subunit at a concentration of 20 and 2 ng. N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibodies did not recognize any specific proteins from untransfected HEK293 cells (Fig. 1*B*, lane 1). However, we observed specific signals of the expected size for the  $\rho 1$  subunit of GABA<sub>C</sub> receptors in HEK293 cells 24 h after adeno- $\rho$  infection at two different titres, 2 and 10 PFU cell<sup>-1</sup> (Fig. 1*B*, lanes 2 and 3, respectively).

Figure 1*C* illustrates current activated by GABA application (10  $\mu\text{M}$ ) in the presence of bicuculline (30  $\mu\text{M}$ ) from HEK293 cells 24 h after adeno- $\rho$  infection. The GABA-evoked current showed no desensitization, had a linear current–voltage relationship, and was insensitive to bicuculline (not shown). The time constant of decay upon GABA removal (deactivation) was  $8 \pm 1$  s (Fig. 1*C*,  $n = 6$ ). The GABA<sub>C</sub> receptor antagonist 3-APA (300  $\mu\text{M}$ ) completely and reversibly blocked this current ( $n = 3$ ). Figure 1*D* is the mean dose–response relationship best fitted with the Hill equation yielding an  $\text{EC}_{50}$  of  $1 \pm 0.3$   $\mu\text{M}$ , and a Hill coefficient of  $2.6 \pm 0.4$  ( $n = 4$ ).

These properties of recombinant  $\rho 1$  GABA<sub>C</sub> receptors after adeno- $\rho$  infection are indistinguishable from those of  $\rho 1$  receptors in HEK293 cells after standard transfection protocols (Filippova *et al.* 1999).

### Pharmacological properties of current recorded from hippocampal neurons in culture before and after adeno- $\rho$ infection

Before expressing  $\rho 1$  subunits, we first characterized the native ligand-activated current in uninfected neurons. Based on these studies, we divided uninfected neurons into three groups according to the properties of the ligand-activated current (Fig. 2*Ai*–*iii*). The first group (Fig. 2*Ai*) represented about 14% (6/44 cells) of the cultured neurons tested and had GABA<sub>A</sub> receptors; that is, a GABA-activated current which was inhibited by bicuculline and had strong desensitization at high GABA concentrations (> 100  $\mu\text{M}$ ). The second group (Fig. 2*Aii*) represented 63% (28/44 cells) of the neurons and had GABA<sub>A</sub> receptors similar to group i, but also exhibited a glutamate-activated current. Note that in both groups, GABA (10  $\mu\text{M}$ ) in the presence of bicuculline (30  $\mu\text{M}$ ) did not activate a current, confirming the absence of native



**Figure 2.** Characteristics of ligand-activated current from hippocampal neurons in culture before and after adeno- $\rho$  infection

*Ai*–*iii*, examples of ligand-activated currents from uninfected neurons at a holding potential of  $-50$  mV. Note that GABA (10  $\mu\text{M}$ ) in the presence of bicuculline (Bicuc; 30  $\mu\text{M}$ ) did not activate current from group i and ii neurons, but evoked a small current from group iii neurons. *B*, 3-APA (300  $\mu\text{M}$ ) did not block the bicuculline-insensitive current from neurons in group iii. SCH 50911 (SCH; 50  $\mu\text{M}$ ) was present to block GABA<sub>B</sub> receptors. *C*, the bicuculline-insensitive current from group iii neurons had a linear current–voltage relationship. *D*, examples of ligand-activated currents from neurons after adeno- $\rho$  infection. Note that GABA (10  $\mu\text{M}$ ) in the presence of bicuculline (30  $\mu\text{M}$ ) evoked a GABA<sub>C</sub>-like current. *E*, 3-APA (300  $\mu\text{M}$ ) completely and reversibly blocked the bicuculline-insensitive current that appeared after adeno- $\rho$  infection. *F*, dose–response relationship of bicuculline-insensitive current after adeno- $\rho$  infection. Glyc, glycine; Glut, glutamate.

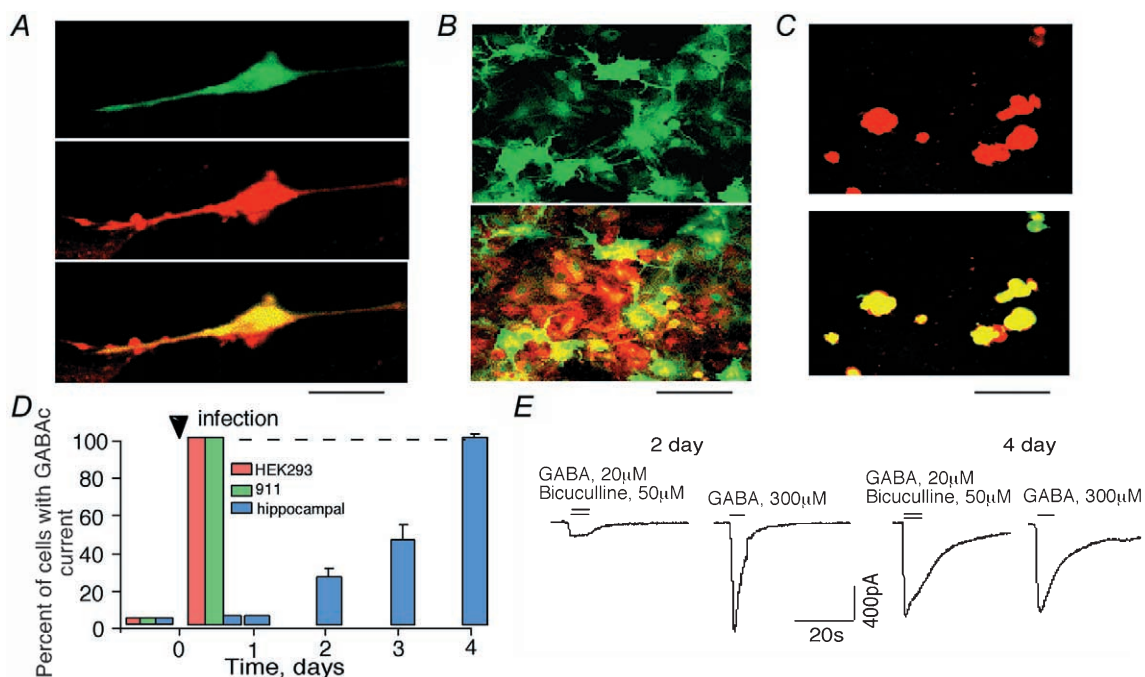
GABA<sub>C</sub>-like receptors. The third group of cells represented 23% of the population (10/44 cells). In addition to the GABA<sub>A</sub> and glutamate currents as in group ii, these neurons contained a glycine-activated current (Fig. 2Aiii). Moreover, GABA (10 μM) in the presence of bicuculline (30 μM) induced a current with an amplitude of 40–100 pA, a linear current–voltage relationship and fast deactivation ( $\tau < 1$  s; Fig. 2C). Unlike a GABA<sub>C</sub> receptor current, this current was not blocked by the GABA<sub>C</sub> competitive antagonist 3-APA (300 μM; Fig. 2B). In fact, we observed a slight potentiation of this current in the presence of 3-APA and the GABA<sub>B</sub> antagonist SCH 50911. A similar potentiation was observed with 3-APA alone (data not shown). Our results suggest that rat hippocampal neurons do not express a classic GABA<sub>C</sub>-like current, although we have not yet identified the receptors responsible for the bicuculline-insensitive, GABA-activated current in neurons from group iii.

Two to three days after adeno- $\rho$  infection, GABA (10 μM), in the presence of bicuculline (30 μM), induced a current with a linear current–voltage relationship, no

desensitization and a slow deactivation rate ( $\tau = 6 \pm 2$  s,  $n = 24$ ) in all three types of cell. Figure 2D shows currents from a type ii neuron after infection. Figure 2E demonstrates that 3-APA (300 μM) completely and reversibly blocked the bicuculline-insensitive GABA-activated current ( $n = 8$ ), and the GABA agonist TACA (10 μM) evoked a current with the same amplitude as GABA (10 μM) with or without bicuculline (30 μM). To estimate the dose–response relationship, we applied GABA at different concentrations in the presence of bicuculline (30 μM; Fig. 2F). The dose–response relationship was best fitted with a single Hill equation yielding an EC<sub>50</sub> of  $1 \pm 0.4$  μM, and a Hill coefficient of  $2.2 \pm 0.4$  ( $n = 4$ ). These results confirm that recombinant  $\rho 1$  receptors were expressed in hippocampal neurons after adeno- $\rho$  infection, and had similar properties to  $\rho 1$  receptors after infection of HEK293 cells (Fig. 1C and D).

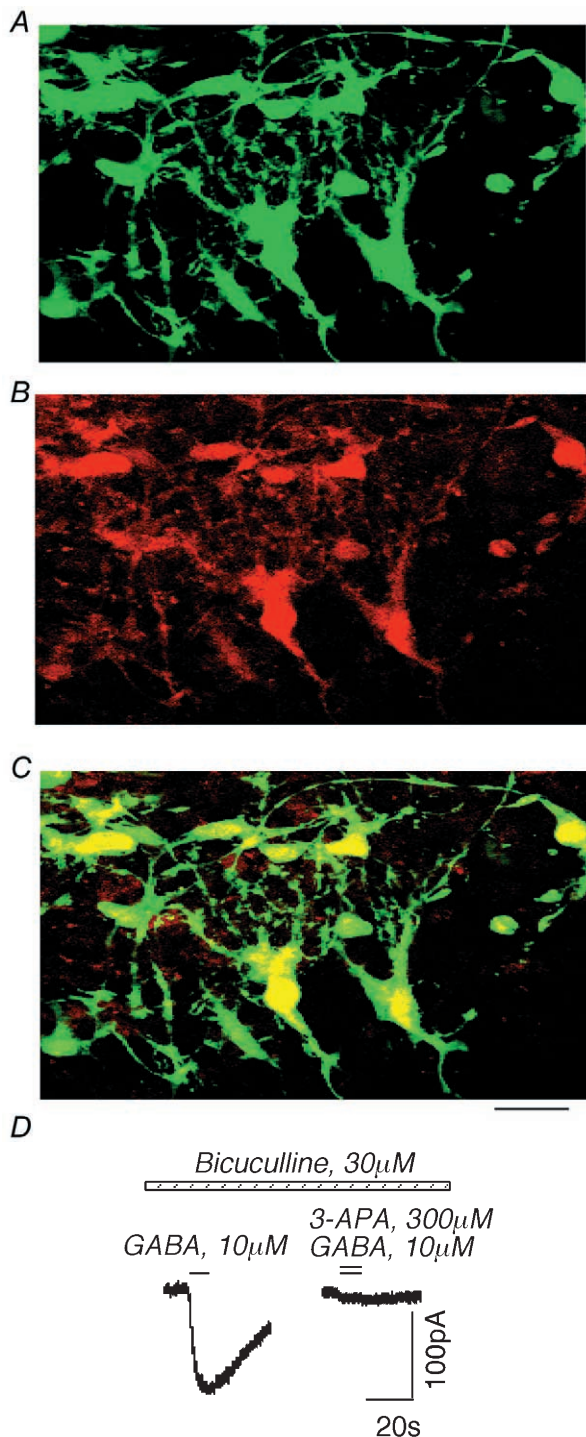
#### Level and time course of expression of recombinant $\rho 1$ GABA<sub>C</sub> receptors

To estimate the percentage of cells that can be infected with adenovirus, we infected HEK293 cells, 911 cells, and hippocampal neurons with an adenovirus containing GFP



**Figure 3.** Level and time course of expression of  $\rho 1$  GABA<sub>C</sub> receptors in neurons and in HEK293 and 911 cell lines after adeno- $\rho$  infection

*A*, cultured neurons were infected with adeno-GFP and visualized with the red fluorescent dye Ro31-8222. Images of a single neuron using a confocal scanning microscope. The bottom image shows the merging of the green and red channels. *B*, same as in *A* but a group of neurons is shown. *C*, images of HEK293 cells after adeno-GFP infection and treatment with Ro31-8222. Note the 100% co-localization of red and green fluorescence. Scale bars: 50 μm in *A* and *C*, 100 μm in *B*. *D*, percentage of cells expressing recombinant  $\rho 1$  receptors after adeno- $\rho$  infection of HEK293 and 911 cell lines and hippocampal neurons over time. *E*, examples of GABA-activated currents on the second and fourth days after adeno- $\rho$  infection of hippocampal cells. Note that on the fourth day, the GABA<sub>A</sub> current was greatly diminished. Only a GABA<sub>C</sub>-like current with the characteristic slow decay time was present.



**Figure 4.** Recombinant  $\rho 1$  receptors expressed in cultured hippocampal slices after adeno- $\rho$  infection

A–C, images of cultured hippocampal slices after co-infection with adeno-GFP and adeno- $\rho$  using a confocal scanning microscope. A, GFP fluorescence. B, staining with N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibodies (red). C, co-localization of GFP and  $\rho 1$ . Scale bar, 50  $\mu\text{m}$ . D, example of a recombinant GABA<sub>C</sub> current in the slice after adeno- $\rho$  infection. The current was blocked by 3-APA, an antagonist of GABA<sub>C</sub> receptors.

(adeno-GFP). For cell visualization, the membrane-permeable red fluorescent dye Ro31-8222 was added to the external solution 5 min prior to examination. Figure 3A (left) shows a dissociated hippocampal neuron infected with adeno-GFP (top, green fluorescence) and treated with Ro31-8222 dye (middle, red fluorescence). The two images are merged in the bottom panel of Fig. 3A. Confocal scanning microscopy revealed that 70–90% of the neurons in the hippocampal culture had green fluorescence and, thus, this percentage of cells could be potentially targeted by adenovirus (Fig. 3B). In the case of the HEK293 and 911 cell lines, all cells had green fluorescence and showed a cytopathic effect (Fig. 3C).

To estimate the percentage of infectible neurons that express recombinant  $\rho 1$  GABA<sub>C</sub> receptors, we co-infected neuronal cells with both adeno-GFP and adeno- $\rho$ . From 12 h to 4 days after infection, we analysed the current amplitude of recombinant  $\rho 1$  GABA<sub>C</sub> receptors activated by GABA (20  $\mu\text{M}$ ) in the presence of bicuculline (50  $\mu\text{M}$ ) and found that the amplitude of the GABA<sub>C</sub> current increased over this time course. Four days after infection, 100% of the infected cells (about 90% of all neurons) contained a GABA<sub>C</sub> current (Fig. 3D). In the HEK293 and 911 cell lines, 100% of the cells expressed recombinant  $\rho 1$  GABA<sub>C</sub> receptors 12 h after infection (Fig. 3D).

Figure 3E presents examples of GABA<sub>C</sub>- and GABA<sub>A</sub>-activated currents in the hippocampal neurons 2 and 4 days after infection. The amplitude of the recombinant GABA<sub>C</sub> current on the second day of infection was 100–400 pA, which was 4- to 10-fold less than that of the typical GABA<sub>A</sub> current (1–2 nA). However, 4 days post-infection, the GABA<sub>C</sub> current amplitude reached 0.8–1.5 nA, while the GABA<sub>A</sub> current decreased to <300 pA. The mean ratio between the GABA<sub>C</sub> and GABA<sub>A</sub> current amplitudes was  $0.16 \pm 0.05$  ( $n = 10$ ) and  $8 \pm 1$  ( $n = 10$ ) on the second and fourth days post-infection, respectively. We did not observe a significant change in the amplitude of the glutamate-activated current after  $\rho 1$  receptor expression ( $290 \pm 70$  and  $210 \pm 40$  pA before and at 4 days post-adeno- $\rho$  infection, respectively;  $n = 5$ ).

We also observed a decrease in the expression level of  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors transfected into HEK293 cells following infection with adeno- $\rho$ . Seventy-two hours after transfection and 12 h after adeno- $\rho$  infection, the amplitude of the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> current was  $335 \pm 150$  pA ( $n = 5$ ) as compared to  $1486 \pm 600$  pA ( $n = 5$ ) without adeno- $\rho$  infection. A qualitatively similar finding was observed when, rather than adeno- $\rho$  infection, HEK293 cells were transfected with both  $\rho 1$  GABA<sub>C</sub> and  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors. In this case, the GABA<sub>A</sub> current was  $100 \pm 80$  pA ( $n = 4$ ) as compared to  $1486 \pm 600$  pA ( $n = 5$ ) with  $\alpha 1\beta 2\gamma 2$  expression alone.

### Recombinant $\rho 1$ GABA<sub>C</sub> receptors expressed in cultured hippocampal slices after adeno- $\rho$ infection

Cultured hippocampal slices were co-infected with both adeno-GFP and adeno- $\rho$ . Three days after infection, slices were fixed and primary N-terminal  $\rho 1$  GABA<sub>C</sub> receptor and secondary Texas Red-conjugated antibodies were used for visualization of  $\rho 1$  GABA<sub>C</sub> receptors. Control non-infected slices did not exhibit specific antibody staining (not shown). However, we observed bright red fluorescence confirming the presence of  $\rho 1$  receptors on the cell surface 3 days after adeno- $\rho$  infection (Fig. 4B). Moreover, we observed a strong correlation between green and red fluorescence (Fig. 4A–C) suggesting that adeno-GFP and adeno- $\rho$  infected the same neurons.

To confirm the functional expression of  $\rho 1$  receptors, we employed the patch-clamp technique in the whole-cell recording configuration. In uninfected slices, we did not observe a native GABA<sub>C</sub>-like current (5/5 cells). However, in adeno- $\rho 1$ -infected neurons, GABA (10  $\mu$ M) in the presence of bicuculline (30  $\mu$ M) induced a current with a linear current–voltage relationship, and slow decay time ( $\tau > 10$  s; 4 of 4 cells). As expected for GABA<sub>C</sub> receptors, the current was reversibly blocked by 3-APA (300  $\mu$ M;  $n = 4$ ; Fig. 4D). Thus, recombinant  $\rho 1$  receptors were expressed in hippocampal slices after adeno- $\rho$  infection.

## DISCUSSION

We have demonstrated that recombinant  $\rho 1$  GABA<sub>C</sub> receptors could be expressed in hippocampal neurons after adeno- $\rho$  infection and these receptors exhibited properties similar to those of recombinant  $\rho 1$  GABA<sub>C</sub> receptors previously described in HEK293 cells (Filippova *et al.* 1999). Furthermore, the hippocampal neurons expressed  $\rho 1$  receptors regardless of their existing complement of ligand-activated receptors.

The distribution of  $\rho 1$  subunits has been identified by RT-PCR and *in situ* hybridization in the retina, superior colliculus, dorsal lateral geniculate nucleus and visual cortex (Boue-Grabot *et al.* 1998). In our experiments, we did not observe specific immunostaining of hippocampal neurons using N-terminal  $\rho 1$  GABA<sub>C</sub> receptor antibodies, suggesting either the absence, or very low levels, of native GABA<sub>C</sub> receptors in the hippocampus. Overall, based on patch-clamp experiments and immunostaining, we conclude that  $\rho 1$  GABA<sub>C</sub> receptors are not evident in the rat hippocampus at P4–P8. The presence of other subunit combinations ( $\rho 2$ ,  $\rho 3$ ) of GABA<sub>C</sub> receptors cannot be confirmed or eliminated. Considering the native bicuculline-insensitive current that was not blocked by 3-APA (Fig. 2Aiii), it is possible that GABA<sub>C</sub> receptor subunits interact with GABA<sub>A</sub> or glycine subunits, and form GABA-activated receptors with unexpected characteristics.

An interesting observation in our study was that expression of  $\rho 1$  GABA<sub>C</sub> receptors after adeno- $\rho$  infection of hippocampal neurons diminished functional expression of GABA<sub>A</sub> receptors. Functional coassembly of the  $\rho 1$ (T314A) GABA<sub>C</sub> subunit with the  $\gamma 2$  GABA<sub>A</sub> receptor subunit was recently confirmed (Pan *et al.* 2000). Moreover, the authors observed a dramatic decrease in the functional expression of GABA<sub>A</sub> and glycine receptors after co-expression with  $\rho 1$  GABA<sub>C</sub> receptors in *Xenopus* oocytes. It is possible that in native neurons, an interaction of recombinant  $\rho 1$  subunits with native  $\gamma 2$  GABA<sub>A</sub> subunits could replace GABA<sub>A</sub> receptors with  $\rho 1$ – $\gamma 2$  chimeric receptors. If this occurs, the interaction would not be evident functionally as the proposed wild-type  $\rho 1$ – $\gamma 2$  receptor has properties indistinguishable from those of  $\rho 1$  alone (Pan *et al.* 2000). Another possibility for the decrease in the GABA<sub>A</sub> receptor current after expression of GABA<sub>C</sub> receptors is that the GABA<sub>C</sub> receptors monopolize the translational machinery of the cells. This explanation seems unlikely, since the amplitude of the glutamate-activated current was unchanged after GABA<sub>C</sub> receptor expression.

During the review of our manuscript, a report appeared that also demonstrated expression of GABA<sub>C</sub> receptors by infection with an adenovirus containing the  $\rho 1$  subunit (Cheng *et al.* 2001). In addition to documenting functional expression, the authors demonstrated that expression of GABA<sub>C</sub> receptors eliminated neuronal hyperactivity and delayed the neuronal death induced by chronic blockade of glutamate receptors. One interesting difference between the two studies was that these authors noted an increased expression of GABA<sub>A</sub> receptors after infection with the  $\rho 1$  subunit as opposed to our observed decrease in GABA<sub>A</sub> expression. A possible explanation for this difference could be the choice of promoter. While we employed a CMV promoter, Cheng *et al.* (2001) used a promoter from Rous sarcoma virus (RSV). The promoter affects cell-type specificity, temporal patterns of expression and absolute expression levels (Smith *et al.* 2000). Comparison of  $\beta$ -galactosidase or GFP expression under the control of different viral promoters in hippocampal neurons demonstrated a high expression level in pyramidal neurons and low expression in granule cells with CMV with the opposite pattern with the RSV promoter (Smith *et al.* 2000). Furthermore, expression under the CMV promoter peaked rapidly and remained high, whereas the RSV promoter produced lower levels of  $\beta$ -galactosidase that began to decrease after several days in culture. These findings are confirmed with  $\rho 1$  as the highest level of expression Chen *et al.* (2001) could obtain with the RSV promoter without observing gross morphological abnormalities was an infection of 10–20% of the cells, whereas we estimated ~70–90% of the cells expressed GABA<sub>C</sub>-like currents with the CMV promoter. The choice of the expression vector could be an important consideration in the control of the levels of GABA<sub>C</sub>

receptors as well as the desired spatial and temporal expression pattern within the central nervous system.

The successful example of the employment of adenovirus in the treatment of cystic fibrosis as well as carcinomas of various organs, including the lung, bladder, ovary and liver, is already well documented (Wilson, 1995; Eck *et al.* 1996). The high infectional capabilities of adeno- $\rho$  with respect to hippocampal neurons opens the way for gene therapy in the treatment of CNS-related diseases such as epilepsy. If the goal is to decrease neuronal excitability, the delivery of GABA<sub>C</sub> receptors composed of  $\rho 1$  subunits may be an optimal choice for several reasons. First, they have a higher sensitivity for GABA compared to GABA<sub>A</sub> receptors. Second,  $\rho 1$  receptors do not desensitize and they demonstrate a slow rate of deactivation upon agonist removal. Both of these factors could increase the efficiency of tonic inhibition of neurons in the presence of low concentrations of extracellular GABA. Finally, the structure–function relationship of  $\rho 1$  receptors has been well characterized allowing the use of custom-made  $\rho 1$  mutants with preferred properties.

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