TOXICOLOGICAL SCIENCES **91(2)**, 540–549 (2006)
doi:10.1093/toxsci/kfj180
Advance Access publication March 31, 2006

Effect of Ketamine on Dendritic Arbor Development and Survival of Immature GABAergic Neurons *In Vitro*

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Received February 14, 2006; accepted March 27, 2006

Ketamine, a noncompetitive antagonist of the N-methyl-D-aspartate type of glutamate receptors, was reported to induce neuronal cell death when administered to produce anesthesia in young rodents and monkeys. Subanesthetic doses of ketamine, as adjuvant to postoperative sedation and pain control, are also frequently administered to young children. However, the effects of these low concentrations of ketamine on neuronal development remain unknown. The present study was designed to evaluate the effects of increasing concentrations (0.01–40 μg/ml) and durations (1–96 h) of ketamine exposure on the differentiation and survival of immature γ-aminobutyric acidergic (GABAergic) interneurons in culture. In line with previous studies (Scallet *et al.*, 2004), we found that a 1-h-long exposure to ketamine at concentrations ≥ 10 μg/ml was sufficient to trigger cell death. At lower concentrations of ketamine, cell loss was only observed when this drug was chronically (> 48 h) present in the culture medium. Most importantly, we found that a single episode of 4-h-long treatment with 5 μg/ml ketamine induced long-term alterations in dendritic growth, including a significant ($p < 0.05$) reduction in total dendritic length and in the number of branching points compared to control groups. Finally, long-term exposure (> 24 h) of neurons to ketamine at concentrations as low as 0.01 μg/ml also severely impaired dendritic arbor development. These results suggest that, in addition to its dose-dependent ability to induce cell death, even very low concentrations of ketamine could interfere with dendritic arbor development of immature GABAergic neurons and thus could potentially interfere with the development neural networks.

Key Words: **dendrite; development; GABA; ketamine; neurotoxicity.**

Pharmacological blockade of N-methyl-D-aspartate (NMDA) type of glutamate receptors as well as the activation of γ-aminobutyric $acid_A$ ($GABA_A$) receptors during the brain growth spurt period, including dendritic development and synaptogenesis, lead to increased apoptotic degeneration of immature neurons in the developing brain (Ikonomidou *et al.*, 1999, 2000). Since the majority of currently used anesthetics inhibit NMDA receptors and/or increase the inhibitory tone of the central nervous system (CNS) via the stimulation of $GABA_A$ receptors, they might exert adverse effects on neuronal development. Indeed, exposure of rat embryos to halothane *in utero* was reported to alter dendritic growth of developing neurons (Uemura *et al.*, 1985), and this was associated with learning impairments during the postnatal period (Levin *et al.*, 1991). In line with these results, a combination of midazolam–nitrous oxide–isoflurane anesthesia has recently been shown to trigger widespread apoptotic cell death in the brain of 7-day-old rat pups (Jevtovic-Todorovic *et al.*, 2003).

Ketamine is a widely used pediatric anesthetic, and renewed interest has recently focused on the use of this agent for the treatment of acute and chronic pain in both pediatric and adult populations (Elia and Tramer, 2005; Himmelseher and Durieux, 2005). Ketamine not only primarily blocks NMDA-mediated neurotransmission by binding noncompetitively to the phencyclidine-binding site of the NMDA receptor (Oye *et al.*, 1992) but also interacts with adenosinergic, monoaminergic, cholinergic, and opioid receptor–mediated signaling pathways (Adams, 1998; Mazar *et al.*, 2005). Similar to the blockade of the NMDA receptor by specific pharmacological agents, exposure of the developing rat brain to ketamine was reported to increase neuronal apoptosis (Hayashi *et al.*, 2002; Ikonomidou *et al.*, 1999; Young *et al.*, 2005). Ketamine also induces neurodegeneration in the adult brain after subcutaneous injections (Olney *et al.*, 1989), and it might potentiate cerebrocortical damage induced by nitrous oxide (Jevtovic-Todorovic *et al.*, 2000). Recent observations indicate that relatively mild exposure to ketamine can also trigger apoptotic neurodegeneration in the developing mouse brain (Young *et al.*, 2005). This issue is of particular interest, since ketamine at subanesthetic concentrations is increasingly used as an adjuvant to multimodal pain therapy and sedation in pediatric anesthesia practice (Lin and Durieux, 2005).

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Neuronal apoptosis is not the only parameter to be considered in evaluating potential adverse effects of ketamine or other anesthetics on neuronal development. It is now well established that interference with the finely tuned molecular mechanisms, guiding the formation of neuronal dendritic arbors in the developing brain, can lead to persistent dysfunctions of the CNS (Webb *et al.*, 2001). Dendrites represent the primary sites of synaptic contacts in developing neurons, and we have recently shown that exposure of developing GABAergic neurons to low concentrations of propofol, while not affecting survival, alters significantly dendritic development of these cells (Vutskits *et al.*, 2005).

Based on previous experimental studies characterizing blood levels of ketamine associated with neurotoxicity in perinatal rat pups (Scallet *et al.*, 2004) as well as on plasma levels of this drug necessary to induce anesthesia in humans (Malinovsky *et al.*, 1996; Weber *et al.*, 2004), the present study was designed to assess the effects of ketamine on neuronal dendritic arbor development using our previously described cell culture model, where isolated neuroblasts from the postnatal subventricular zone (SVZ) survive and differentiate into GABAergic interneurons (Gascon *et al.*, 2005).

MATERIALS AND METHODS

Cell culture and reagents. After obtaining approval from the Animal Care Committee of the University Medical Center, cell cultures were prepared from newborn (postnatal day 0) Sprague-Dawley rats. Animals were sacrificed by decapitation, and the brain was carefully removed and transferred into an ice-cold Hank's magnesium- and calcium-free solution. Two coronal cuts were then made to expose the anterior horn of the lateral ventricles, and the SVZ was microdissected. The small tissue pieces obtained were dissociated mechanically and digested with trypsin (Invitrogen Life Technologies, Paisley, United Kingdom) for 15 min at 37°C. The trypsin reaction was stopped with 1 ml of cold fetal calf serum, and cells were recovered after 10 min of centrifugation at 300 × g. To eliminate cell debris, the pellet was resuspended into 1 ml of phosphate-based saline (PBS) and layered onto a 22% Percoll (Amersham Pharmacia, Little Chalfont, United Kingdom) in PBS and centrifuged 10 min at 500 × g. Cells were washed three times with culture medium before plating onto polyornithine (Sigma, St Louis, MO)–coated coverslips in 35 mm petri dishes (Falcon, Plymouth, United Kingdom). Seeding density was 5000 cells/cm^2. Cells were cultured in neurobasal medium (Invitrogen Life Technologies) supplemented with 2% B27 (Invitrogen Life Technologies), 200mM L-glutamine (Invitrogen Life Technologies), and 1mM Na pyruvate (Sigma). Under these conditions, cells readily survived and developed as GABAergic neurons for up to 12 days in culture.

To test the effects of ketamine on neuronal survival and development, cultures were exposed to ketamine (Ketalar, Parke-Davis, Berlin, Germany) and the NMDA receptor antagonist MK 801 (100μM; Tocris, Bristol, United Kingdom). The amount and the duration of ketamine treatment are indicated in detail in each experiment. Briefly, for each experimental protocol, cells were kept in the above-mentioned culture medium for 24 h following seeding, allowing attachment to the polyornithine substrate. Ketamine as well as MK 801 were then administered into the culture medium for a defined period as indicated in the "Results" section and corresponding figures. To remove drugs from the culture medium, cultures (control and treated) were washed three times with warm (37°C) Neurobasal medium and then continued to be cultured in the presence of Neurobasal medium before analysis according to experimental protocols.

Immunocytochemistry. Cells were fixed with cold (4°C) paraformaldehyde 4% in phosphate buffer (pH 7.4). Then, they were rinsed three times in PBS and incubated overnight at 4°C with the primary antibody diluted in PBS 0.5% containing bovine serum albumin (BSA) (0.3%), and Triton X-100. The mouse monoclonal antibody directed against β-tubulin isotype III (Sigma, 1:400 dilution) was used to identify neurons. Bound antibodies were revealed with rhodamine- or fluorescein-conjugated sheep anti-mouse IgG (Boehringer, Mannheim, Germany; dilution 1:40 for rhodamine and 1:80 for fluorescein) diluted in PBS containing 0.5% BSA. Immunostained cultures were examined with an Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

TUNEL assay. Apoptotic cells were revealed using the TUNEL assay. Briefly, cultures were rinsed with PBS and incubated for 15 min with the TUNEL buffer (Tris 30mM, Na cacodylate 140mM, and cobalt chlorid 1mM). Then, the enzyme terminal transferase (Roche, Mannheim, Germany) 0.3 U/μl and the labeled dUTP 6μM were applied for 90 min at room temperature. The reaction was stopped with 2× SSC (sodium citrate buffer) and cultures washed again with PBS. 7′-Fluorescein dUTP (Roche, Mannheim, Germany) was chosen as a label. The fluorescent labeling allowed quantifying apoptotic cells under a fluorescent microscope (Axiophot, Carl Zeiss, Jena, Germany). For colocalization with cell-specific markers, cultures were subjected to immunohistochemistry prior to the TUNEL assay.

Cell counts, statistical analysis, image acquisition, and processing. Cultures were examined using an Axiophot fluorescence microscope (Carl Zeiss). Cells were counted with the help of a square grid placed into the ocular of the microscope. Using a ×40 objective, the grid area represented 0.16 mm^2. On each coverslip, 30 samples (= square grids) were randomly taken and then samples pooled (i.e., total surface measured per coverslip was 4.8 mm^2). Data are expressed as the number of neurons/mm^2 ± SEM and reflect the results obtained form at least three independent experiments.

For quantitative analysis of dendritic arbors, cells were stained with the monoclonal GABA antibody and photographed. Before the analysis, brightness and contrast were optimized with Adobe Photoshop program (Adobe Systems Incorporated, San Jose, California). The following parameters of dendritic shape and extent were then determined: number of primary dendrites (PDs), length of dendrites, and the number of dendritic branches. Total dendritic length (TDL) was measured drawing all visible processes with Scion software (Scion Corporation, free download at http://www.scioncorp.com/frames/fr_download_now.htm). The remaining parameters were manually scored on the image. Processes shorter than 5 μm were excluded from the analysis. Values were expressed as means ± SEMs and analyzed for statistical significance. Differences between groups were first discriminated by one-way ANOVA and then the unpaired t test was performed, where t was corrected for multiple comparisons against the untreated group using the Bonferroni test. $^*p < 0.05$ compared with the untreated control group.

RESULTS

Dose- and Exposure Time–Dependent Effects of Ketamine on GABAergic Neuronal Survival

To study the effect of ketamine on neuronal differentiation, we took advantage of our previously described culture model that allows tracking quantitatively dendritic arbor development of GABAergic neuronal precursors (Gascon *et al.*, 2005; Vutskits *et al.*, 2005). These cells were shown to express both GABA and NMDA receptors *in vivo* (Carleton *et al.*, 2003), and accordingly, we found that application of GABA as well as glutamate into the culture medium initiated calcium responses in these neurons *in vitro* (data not shown). As ketamine was

reported to trigger apoptosis in the immature brain *in vivo* (Ikonomidou *et al.*, 1999; Young *et al.*, 2005), we first determined the concentration range in which ketamine may induce death of cultured interneurons. We tested the effects of ketamine under two conditions: after a single short-term (1–8 h) treatment and after the continuous presence of ketamine for 24–96 h. In a first series of experiments, cultures at 1 day *in vitro* were exposed to ketamine for 1 h in concentrations ranging from 0.01 to 40 μg/ml (for experimental setup see Fig. 1A). No cell loss was observed at any of these concentrations when cell survival was assessed immediately after this treatment (Fig. 1B). As seen in Figure 1C, in control cultures, neurons survive and start to differentiate under serum-free conditions. In contrast, we observed a marked cell loss as early as 24 h following treatment in cultures where cells were exposed to ketamine at concentrations of $\geq$ 10 μg/ml, and this was further accentuated by the end of the 48th h postexposure (Figs. 1D–1E). Immunocytochemical colocalization of tubulin-β-III–positive pycnotic neurons with the apoptotic marker TUNEL revealed that the nature of this cell death is apoptosis (Fig. 1E, right upper window). Quantitative assessment of cell survival showed a significant decrease ($p < 0.05$) in the number of surviving neurons by the end of the 24th h following a 1-h-long ketamine treatment at concentrations $\geq$ 10 μg/ml, and this cell loss further increased by the end of the second day (Fig. 1G). Thus, a single short-term exposure to 10 μg/ml of ketamine is able to induce delayed apoptotic death of immature neurons. In contrast, exposure of cultures to ketamine at concentrations $\leq$ 5 μg/ml up to 8 h did not trigger apoptosis of GABAergic neurons (not shown). Since one major action of ketamine is the blockade of the NMDA-type glutamate receptors, we also examined cell survival in the presence of the noncompetititve NMDA receptor blocker MK 801 (100μM). The presence of MK 801 up to 8 h in the culture medium did not induce cell death (Figs. 1F–1G).

We next explored the effects of long-term ketamine administration on cell survival and found that ketamine at concentrations as low as 1 μg/ml induced neuronal cell loss when chronically (> 48 h) present in the culture medium (Fig. 2). Similarly, chronic application (> 24 h) of MK 801 (100μM) led to a significant decrease ($p < 0.05$) in the number of neurons (Fig. 2), suggesting that NMDA-dependent mechanisms are involved in this process.

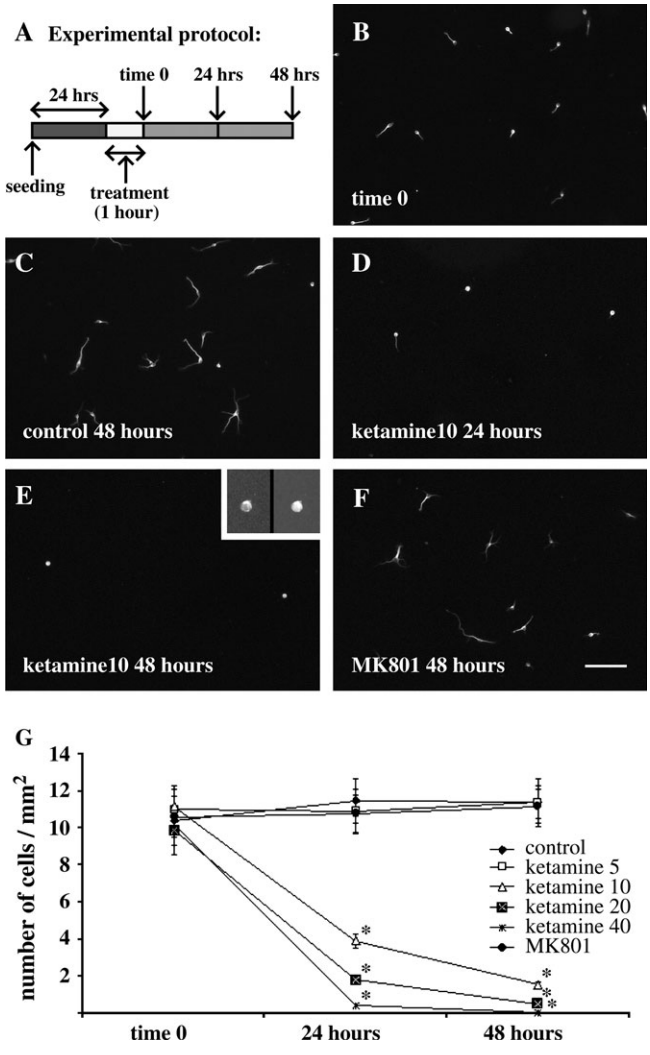

FIG. 1. Short-term (1 h) exposure to ketamine at concentrations of $\geq$ 10 μg/ml triggers apoptosis of developing GABA-positive neurons. (A) Experimental protocol. (B) Twenty-four hours after seeding, isolated neuroblasts exhibit an immature morphology under serum-free conditions. (C) Neuroblasts survive and start to differentiate in control cultures. (D) Twenty-four hours following ketamine (10 μg/ml for 1 h) exposure, the number of surviving neurons decreases. (E) The number of neurons further decreases by the 48th h following ketamine treatment. Right upper window: colocalization of tubulin-β-III–positive neurons (left) with the TUNEL labeling (right) revealed that the nature of this cell death is apoptosis. (F) Short-term treatment with the noncompetitive NMDA antagonist MK 801 (100μM) does not affect neuronal survival and differentiation. (G) Quantitative analysis of cell survival following a short-term (1 h) exposure of developing neurons to ketamine and MK 801(100μM). In photomicrographs (B–F), cells were stained with the neuron-specific marker tubulin-β-III antibody. Correction bar (B–F): 200 μm. In (G), results are presented as mean ± SEM; $n = 3$ independent experiments for each time point and each treatment expressed. Values are expressed as the number of neurons/mm^2. *$p < 0.05$ compared with the untreated control group.

Ketamine at Nonapoptotic Concentrations Can Impair Dendritic Arbor Development of GABAergic Neurons

The relationship between the dendritic morphology of a neuron and its function is well established (Yuste and Tank, 1996), and increasing evidence suggests that even subtle alterations of dendritic arbor development can lead to permanent CNS dysfunction (Webb *et al.*, 2001). We thus examined whether exposure to ketamine at non–cell death-inducing concentrations can alter dendritic growth and arborization pattern of developing GABAergic neurons. To assess the effect of short-term ketamine treatment on dendritic development, cultures were grown for 24 h in serum-free medium and then exposed to ketamine at concentrations ranging from 1 to 5 μg/ml

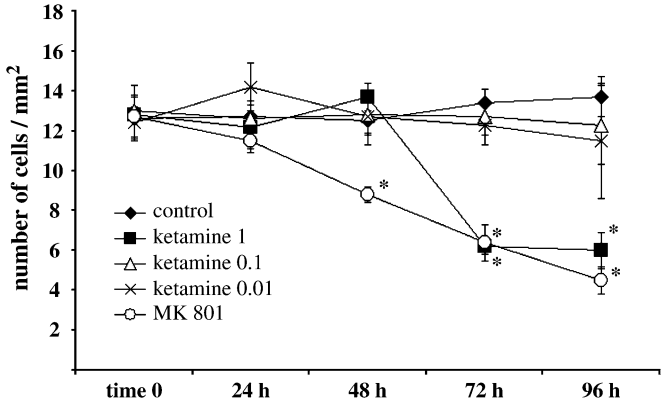

FIG. 2. Effect of continuous exposure to ketamine and MK 801 on neuronal survival. Twenty-four hours after seeding (time 0), ketamine (0.01, 0.1 and 1 μg/ml) and MK 801 (100μM) were added to the culture medium, and neuronal survival was assessed in corresponding sister cultures every 24 h up to 4 days. Results are presented as mean ± SEM; $n = 3$ independent experiments for each time point and each treatment expressed. Values are expressed as the number of neurons/mm^2. *$p < 0.05$ compared with the untreated control group.

for 1–8 h. Cells were then washed extensively to remove ketamine, and serum-free medium was added again for an additional 24–96 h (for experimental setup see Fig. 3A). Corresponding sister cultures were then fixed and analyzed at 0, 48, and 96 h. To quantify the impact of ketamine treatment, we measured three parameters: (1) TDL; (2) the number of PDs, defined as those arising from the cell body; and (3) the number of branching points (BPs).

As seen in Figure 3, 24 h after seeding (time 0), all neurons in culture exhibited a rather immature morphology with short dendritic processes. In control, placebo-treated groups, neurons progressively differentiated and developed a highly complex arborization pattern by time (Fig. 3B). Quantitative analyses revealed an approximately 10-fold increase in TDL during the first 5 days in culture, and this was accompanied by a significant ($p < 0.05$) augmentation of arbor complexity in terms of PD and BP (Fig. 4). The 1-h-long treatment with ketamine up to concentrations of 5 μg/ml did not affect any aspects of subsequent dendritic development (not shown). In contrast, a 4-h-long exposure of neurons to ketamine at a concentration of 5 μg/ml led to reduced dendritic growth and arbor complexity compared to placebo-treated groups or cultures exposed to lower doses of this agent (Figs. 3–4). In this group, a significant ($p < 0.05$) impairment in subsequent dendritic growth and arborization could be detected as early as 48 h following ketamine exposure, and this difference was further accentuated by the end of the 96th h in culture (Fig. 4). Figure 5 shows the impact of an 8-h-long ketamine treatment on various aspects of ulterior dendritic growth in culture. We found a significant difference ($p < 0.05$) in TDL in cultures exposed to 2 μg/ml of ketamine as early as 48 h following treatment, and this difference was further accentuated by the end of the 96th h. Additionally, while the number of PDs did not differ between groups 96 h following ketamine treatment (2 μg/ml), we found a significant ($p < 0.05$) decrease at this time point in both TDL and arborization pattern, expressed as the number of branch point, compared to controls (Fig. 5). Taken together, these results suggest that even a single short episode of ketamine treatment at non–cell death-inducing concentrations can lead to persistent changes in dendritic development.

We also investigated the effect of long-term ketamine treatment as well as the impact of NMDA receptor blockade on dendritic arbor development of GABAergic neurons at concentrations ranging from 0.01 to 1 μg/ml. We found that dendritic development of GABAergic neurons was severely impaired following chronic exposure even to low concentrations (0.1–0.01 μg/ml) of ketamine (Figs. 6–7). Following the first 24 h in the presence of ketamine (0.01–1 μg/ml), no difference could be detected between control and ketamine-treated cultures in any aspects of dendritic development analyzed (Fig. 7). In contrast, by the end of the second day, both TDL and the number of branch points were significantly decreased in cultures treated with ketamine at concentrations between 0.1 and 1 μg/ml. Importantly, analysis at later time points (72 and 96 h) revealed that continuous administration of ketamine in doses as low as 0.01 μg/ml has a significant deleterious effects on dendritic development (Fig. 7). Taken together, these data indicate that very low doses of ketamine are sufficient, when chronically applied, to reduce dendritic arbor expansion of immature developing GABAergic neurons.

DISCUSSION

Given the widespread use of ketamine in pediatric anesthesia practice, an important question is whether administration of this drug can exert potential adverse effects on the developing CNS. Indeed, increasing evidence suggests that anesthetic as well as subanesthetic doses of ketamine can trigger apoptotic neuronal death in the immature brain (Ikonomidou *et al.*, 1999; Scallet *et al.*, 2004; Young *et al.*, 2005). Here we show that a short-term exposure of developing GABAergic neurons to low concentrations (5 μg/ml) of ketamine, which do not interfere with cell survival, results in altered dendritic growth of these cells. We also demonstrate that chronic administration of ketamine at concentrations as low as 0.01 μg/ml, while not affecting survival, severely alters dendritic development. These results suggest that, in addition to its ability to trigger apoptosis of immature neurons, ketamine may interfere with the fundamental mechanisms that govern dendritic arbor development of immature neurons.

As a complement to *in vivo* experimentation, *in vitro* models are useful tools to draw the attention to the potential adverse effects of pharmacologic agents during development. Here, we used a recently described *in vitro* model where isolated and purified neuronal precursors from the newborn rat SVZ were cultured at a low seeding density and differentiated into GABAergic neurons (Gascon *et al.*, 2005). A major advantage

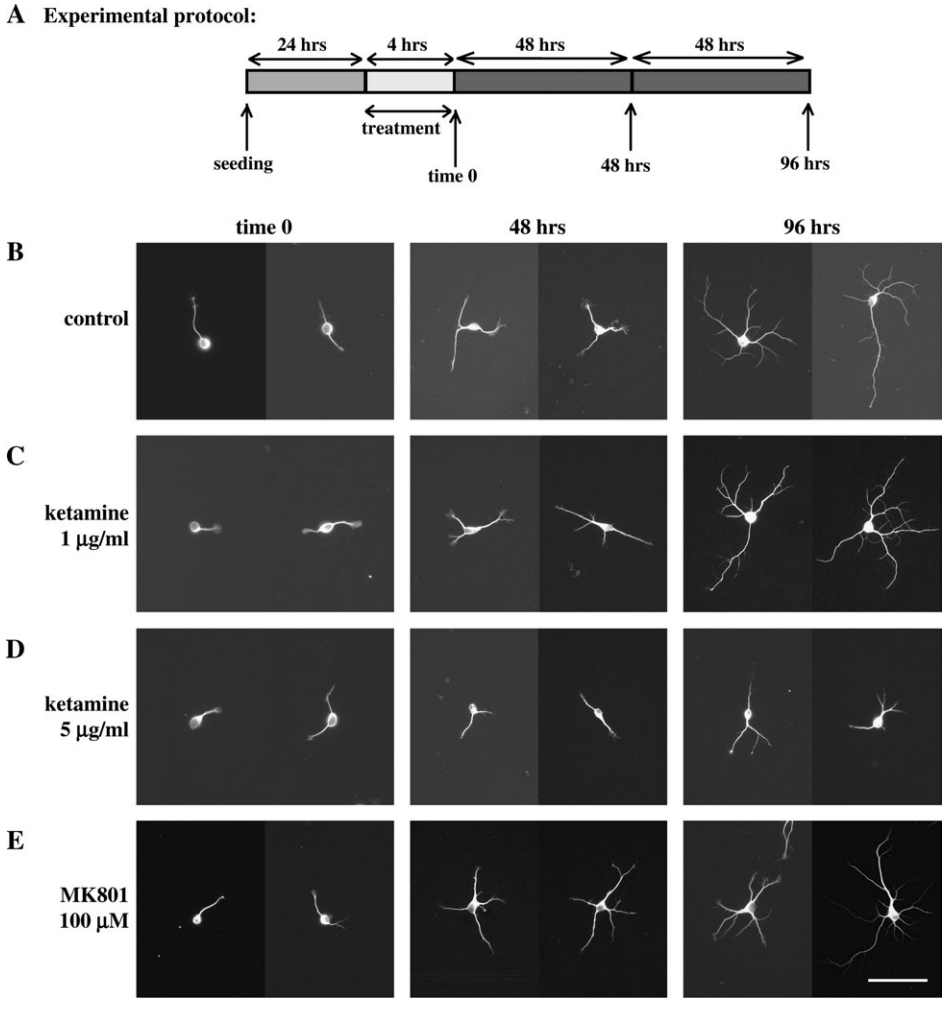

FIG. 3. Effects of short-term (4 h) ketamine and MK 801 treatment on subsequent dendritic development. (A) Experimental protocol. (B) Representative examples of dendritic growth in control, (C) ketamine- (1 µg/ml), (D) ketamine- (5 µg/ml), and (E) MK 801 (100µM)–treated neurons. Note the substantially reduced dendritic arborization after treatment with ketamine at 5 µg/ml. Cells were stained with the neuron-specific marker tubulin-β-III antibody. Correction bar (B–D): 80 µm.

of this model is that, in addition to assessment of cell death, it also allows observing and quantifying the effects of anesthetics on dendritic growth and branching. As proper development of dendrites is essential for the establishment of neuronal circuitry (Chen and Ghosh, 2005) and dendritic morphology plays a critical role in synaptic integration and information processing (Jan and Jan, 2003; Miller and Kaplan, 2003), assessment of morphofunctional parameters describing dendritic arbor development is of utmost importance in terms of evaluating anesthesia-related neurotoxicity.

It is now well established that even subtle alterations of the neuronal dendritic tree can lead to persistent dysfunctions of the CNS without inducing apparent cell loss (Webb *et al.*, 2001). The results presented here suggest that non–cell death-inducing concentrations of ketamine might still impair CNS development by interfering with dendritic growth. We applied this drug to the culture medium at a wide concentration range (0.01–40 µg/ml) including concentrations corresponding to reported plasma levels in previous animal as well as human studies (Malinovsky *et al.*, 1996; Scallet *et al.*, 2004; Weber *et al.*, 2004). Using this approach, we found that even very low concentrations of ketamine (0.01 µg/ml) can induce substantially reduced dendritic growth, when this agent is present continuously in the culture medium. Given that ketamine at low subanesthetic doses is increasingly used for extended time periods as an adjuvant to postoperative sedation and pain control (Albanese *et al.*, 2004; Himmelseher and Durieux, 2005), these *in vitro* findings could be of potential interest for further animal experiments.

One major mechanisms of action of ketamine is the blockade of the NMDA type of glutamate receptors. In agreement with previous observations (Ikonomidou *et al.*, 1999), we found that prolonged exposure to the NMDA receptor antagonist MK 801 increased apoptosis of GABAergic neurons. When applied

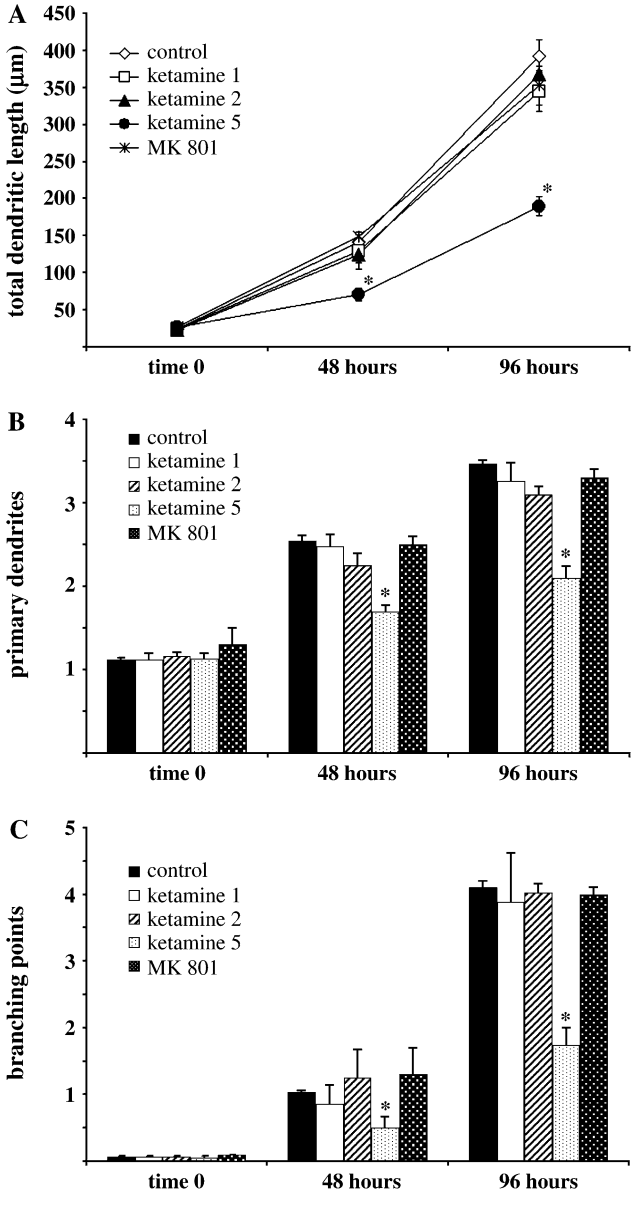

FIG. 4. Quantitative assessment of dendritic development after a 4-h-long ketamine and MK 801 exposure. For experimental protocol, see Figure 3A. Neither exposure to ketamine up to concentrations of 2 µg/ml nor to MK 801 (100µM) resulted in decreased dendritic growth, as expressed by the TDL (A), number of PDs (B), and number of BPs (C). In contrast, this short-term exposure of developing neurons to ketamine at a concentration of 5 µg/ml altered all three of these parameters. Results are presented as mean ± SEM; $n = 3$ independent experiments (three culture dish per experiment, 30 neurons per culture dish) for each time point and each treatment expressed. $^*p < 0.05$ compared with the untreated control group.

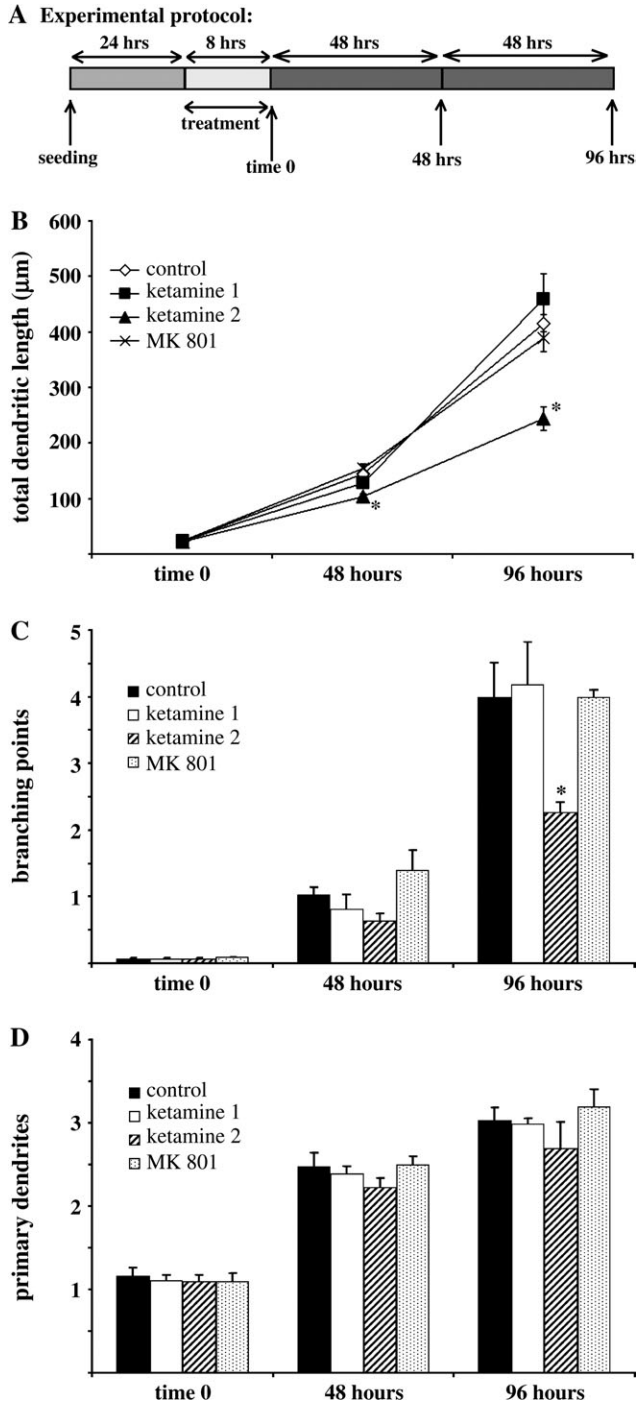

FIG. 5. Quantitative assessment of dendritic development after an 8-h-long exposure to ketamine and MK 801. (A) Experimental protocol. (B) TDL and (C) the number of number of BPs but not that of PDs (D) were significantly altered by the end of the 96th h following an 8-h-long treatment with ketamine at a concentration of 2 µg/ml. In contrast, ketamine at 1 µg/ml and MK 801 (100µM) had no such effect. Results are presented as mean ± SEM; $n = 3$ independent experiments (three culture dish per experiment, 30 neurons per culture dish) for each time point and each treatment expressed. $^*p < 0.05$ compared with the untreated control group.

continuously, the effect of MK 801 was quite similar to the effect of ketamine at a concentration of 1 µg/ml. The observation that long-term exposure to lower doses of ketamine ($\leq$ 0.1 µg/ml) does not induce cell death could be explained by a partial blockade of the NMDA receptor. In the developing human brain, NMDA receptor activity and expression increase

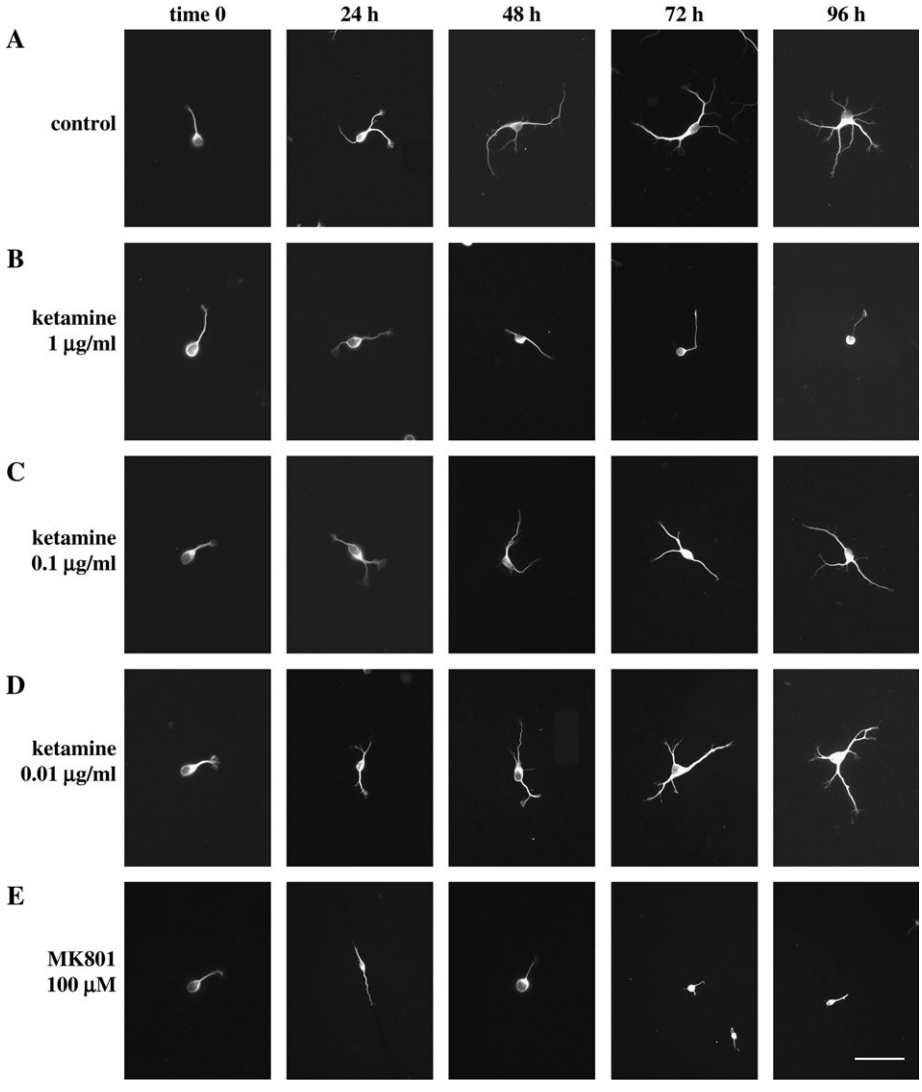

FIG. 6. Effect of long-term ketamine and MK 801 treatment on dendritic development. Twenty-four hours after seeding (time 0), ketamine was applied to the culture medium and left there for up to 96 h. Representative examples of dendritic arbor expansion in (A) control, (B) ketamine- (1 μg/ml), (C) ketamine- (0.1 μg/ml), (D) ketamine- (0.01 μg/ml), and (E) MK 801 (100μM)–treated neurons. Cells were stained with the neuron-specific marker tubulin-β-III antibody. Correction bar (A–D): 85 μm.

in the early infant period, whereas α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionate and kainate receptors are elevated during midgestation and decrease thereafter (Panigrahy *et al.*, 2000). Increasing evidence suggests that signaling through the NMDA receptor complex is important for the maturation and plasticity of developing CNS (Waters and Machaalani, 2004). Pharmacological blockade of NMDA receptors has been shown to reduce dendritic growth rates in the *Xenopus tectum* (Rajan and Cline, 1998), and similar results were obtained in the rodent supraoptic nucleus (Chevaleyre *et al.*, 2002) and in spinal motoneurons (Kalb, 1994). In addition to participating in dendritic sculpting, NMDA receptors also have a direct role in neuronal proliferation (Gould *et al.*, 1994) and migration (Komuro and Rakic, 1993). Parallel to its important physiological role in CNS development, both excessive stimulation and chronic blockade of the NMDA receptor complex have been shown to induce widespread neuronal cell death in the immature brain (Ikonomidou *et al.*, 1999; Portera-Cailliau *et al.*, 1997). In this context, ketamine and other anesthetics, able to modify the highly orchestrated equilibrium of NMDA receptor signaling (Waters and Machaalani, 2004), are potential candidates to hinder normal neuronal development when administered during the brain growth spurt period.

While ketamine is considered to exert its effect primarily through the noncompetitive blockade of the NMDA receptor, we found substantial differences, depending on the experimental protocol, between this anesthetic and the noncompetitive NMDA receptor antagonist MK 801 on neuronal differentiation and survival. The fact that a 1-h-long treatment with

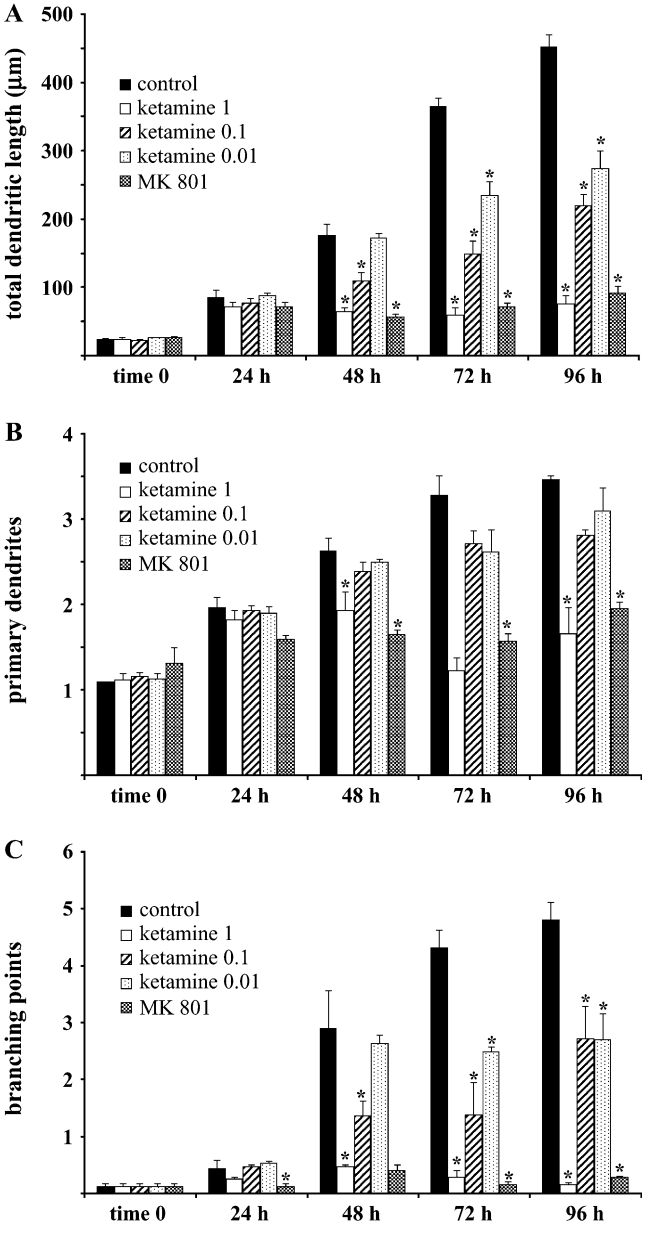

FIG. 7. Quantitative analysis of dendritic development after long-term ketamine and MK 801 treatment. Continuous exposure to low concentrations of ketamine (0.01–1 µg/ml) and MK 801 (100µM) resulted in most cases after 48 h in an altered dendritic development, as expressed by the TDL (A), the number of PDs (B), and the number of BPs (C). Results are presented as mean ± SEM; $n = 3$ independent experiments (three culture dish per experiment, 30 neurons per culture dish) for each time point and each treatment expressed. $^*p < 0.05$ compared with the untreated control group.

ketamine, but not with MK 801, exposure was sufficient to trigger an important apoptosis of GABAergic neurons raises the possibility that, at high-dose regimens, ketamine-induced neurotoxicity is, at least partially, independent of NMDA receptor blockade. These data were further confirmed by experiments showing that exposure of cultures to MK 801 up to 4 h affect neither survival nor differentiation of developing neurons. One plausible explanation of these observations would be that, in addition to the NMDA receptor blockade, ketamine also interacts with a multitude of signaling pathways mediating neurotransmission in the CNS (Adams, 1998). Indeed, ketamine induces release of dopamine, serotonin, and noradrenaline in the brain (Kari *et al.*, 1978; Tso *et al.*, 2004), and recent experimental evidences indicate that this anesthetic also interferes with the reuptake of these amines from the extracellular space by inhibiting monoamine transporters (Nishimura *et al.*, 1998; Tso *et al.*, 2004). It is of interest that excessive accumulation of monoamines has been reported to trigger extensive neurodegeneration in rodents (Bozzi and Borrelli, 2006), and blockade of the serotonin transporter has been shown to reduce the complexity of dendritic arbor architecture of hippocampal pyramidal neurons (McKittrick *et al.*, 2000). Ketamine also induces the release of adenosine from nerve terminals (Mazar *et al.*, 2005), and there is now evidence that adenosine A_{2A} receptors play a permissive role in the metabotropic glutamate receptor–mediated potentiation of NMDA signaling (Tebano *et al.*, 2005). It is thus possible that, in the presence of higher concentrations of ketamine, additive or synergistic effects between these molecular mechanisms and signaling pathways could rapidly initiate dendritic remodeling and/or apoptosis. Alternatively, large doses of ketamine could exert a nonspecific neurotoxic effect.

The functional relevance of our *in vitro* data remains to be determined. Anesthesia-induced neurotoxicity is a highly debated and controversial issue (Anand and Soriano, 2004; Olney *et al.*, 2004; Todd, 2004). Data presented in this study suggest that clinical and subclinical concentrations of ketamine could interfere with dendritic development and thus might lead to long-term impairment of higher-order CNS functions (Webb *et al.*, 2001). However, it is important to note that extrapolation of these *in vitro* results, obtained at the single-cell level, to clinical practice requires caution. An essential next step in addressing this issue will be to determine how neuronal dendritic arbor development is influenced by ketamine exposure in a more complex and physiological environment, using organotypic slice cultures and *in vivo* animal experiments. Also, while the importance of *in vitro* and *in vivo* experiments to study drug safety and efficacy in developing rodents is well established, one cannot fully exclude the possibility of interspecies differences in terms of drug effects (Berde and Cairns, 2000). Indeed, anesthesia-inducing doses of ketamine in rodents appear to be several folds higher those than in humans (Malinovsky *et al.*, 1996; Scallet *et al.*, 2004; Weber *et al.*, 2004). Additionally, except one recent study measuring ketamine blood levels in rats (Scallet *et al.*, 2004), to our knowledge, there is no study available evaluating ketamine pharmacokinetics as well as blood brain partition coefficient in rodents or higher primates. As it would be practically and ethically impossible to establish a dose-response curve of ketamine-induced neurotoxicity in human infants, such experiments should be performed to further elucidate this question.

Observational studies following neonatal and pediatric surgery provide some indirect information about the effect of anesthesia and analgesia on neurological outcome, but the numerous confounding variables of these observations make it difficult to truly assess the effect of exposure to anesthesia (Soriano *et al.*, 2005). To our knowledge, there are currently no studies evaluating the effect of ketamine on short- and long-term neuropsychological outcomes in infants and children. In contrast, recent double-blinded, placebo-controlled data suggest that ketamine impairs learning of spatial and verbal information when administered to healthy adult subjects (Rowland *et al.*, 2005). As dendrites are important morphological substrates underlying such cognitive dysfunctions, the present results demonstrating the effects of ketamine on dendritic arbor development should give further arguments to promote clinical research on this topic. Despite the difficulty to conduct clinical trials in the neonatal and pediatric populations, these future studies would be necessary to bridge the gap between experimental neuroscience and clinical medicine (Todd, 2004).

ACKNOWLEDGMENTS

This work was supported by Swiss National Foundation Grants (Bern, Switzerland) 3100A0-104059/1 to J.Z.K., 32-53863.98 to E.T., and the Anesthesiology Department Fund of the University Hospital of Geneva (Switzerland) to L.V. We wish to thank to Sylvie Chliate (technician, Department of Neuroscience, University of Geneva Medical School, Switzerland) for technical assistance.

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