Adult hippocampal cell birth and death in relation to stress, aging and the vasculature

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Addendum

Gene Expression Patterns in Rat Dentate Granule Cells: Comparison Between Fresh and Fixed Tissue

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RNA from brain tissue (in particular human brain) can often only be extracted from fixed material. As brain tissue is very heterogeneous with regard to cell type, it is important to obtain RNA from small samples of identified cells. The aim of this study was a) to generate expression profiles from small yet homogeneous samples of fixed brain cells in rat and b) to verify the reliability of these profiles by comparing them with expression profiles obtained from single fresh neurons of the same cell type. Samples (n=12) of 50 rat dentate granule cells were isolated, using Laser Microdissection and Pressure Catapulting, from paraformaldehyde fixed, paraffin embedded tissue or from frozen, ethanol fixed tissue. In addition, RNA was extracted under visual control from individual dentate granule cells (n=12) in hippocampal slices, after electrophysiological recording with patch clamp electrodes. Our data show that RNA was successfully extracted from ethanol fixed sections yielding expression profiles highly comparable to those from non-fixed, single granule cells. RNA extraction from paraformaldehyde fixed, paraffin embedded tissue was less reliable. The present approach validates expression profiling from small amounts of fixed neurons as a powerful tool to investigate molecular processes if fresh tissue is not available.
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Introduction

The most common archival post-mortem material available for gene expression studies is paraformaldehyde fixed, paraffin embedded tissue or frozen material. To unravel possible molecular mechanisms underlying (patho) physiological processes, it is of great interest to obtain an intact RNA sample and a reliable gene expression profile from fixed tissues. Technically, though, this has been a great challenge. Gene expression in fixed material has been investigated with different fixatives in various tissues, including the brain (Cummings et al., 2001; Relf et al., 2002; Scheidl et al., 2002; Van Deerlin et al., 2002). Each fixation method was found to have a specific impact on the integrity and yield of mRNA (O'Dell et al., 1998; Srinivasan et al., 2002), probably introducing errors in the interpretation of expression data. Formalin fixed, paraffin embedded tissues in general gave rise to a low yield and rather poor quality of RNA, whereas ethanol fixed tissue yielded a relatively intact RNA gene expression profile (Rupp and Locker, 1988; Stanta and Schneider, 1991; Finke et al., 1993; Foss et al., 1994; Karlsen et al., 1994; Serth et al., 2000; Shibutani et al., 2000; Specht et al., 2001; Macabeo-Ong et al., 2002). More recently, expression profiles derived from fixed materials and frozen samples were extensively compared in a microarray survey (Karsten et al., 2002), including samples from the dentate gyrus (Elliott et al., 2003).

All of these studies needed a large amount of tissue as starting material for the investigation of gene expression. This is a considerable disadvantage in brain tissue, which is usually characterized by a high degree of heterogeneity and in which often only few cells undergo pathophysiological changes. Clearly, there is great need for a reliable method to examine expression of multiple genes in identified fixed brain cells. Initial studies reported on expression patterns in single, fixed neurons (Eberwine et al., 2001; Ginsberg and Che, 2002; Hemby et al., 2002), as discussed elsewhere (Eberwine et al., 2001, 2002). Recently, the laser dissection microscope (LDM) method was developed, which enables quick and easy collection of very small, homogenous cell populations or even single cells from stained and fixed sections (Schutze and Lahr, 1998), to generate gene expression patterns (Goldsworthy et al., 1999). In non-fixed single neurons, the LDM technology was combined with real time, quantitative PCR (Bi et al., 2002; Vincent et al., 2002).

It still needs to be verified, however, if thus generated expression profiles are really reliable, in particular with regard to the effect of fixation. In the present study, we attempted to generate expression profiles of rat dentate granule cells from paraformaldehyde fixed, paraffin-embedded or ethanol fixed tissue, captured by the LDM method. The
reliability of this method was examined by comparing expression profiles from fixed cells with those from tissue still very close to the original, life situation, i.e. non-fixed, single rat dentate granule cells, collected just after functional characterization by whole cell patch clamp recording in hippocampal slices.

**Materials and Methods**

*Collection of dentate granule cells*

In the present study we used archival hippocampal sections (8 μm) from 6 weeks old rats. These sections had been either paraformaldehyde-fixed (4%) by perfusion (Heine *et al.*, 2003), prior to paraffin-embedding (Merck), or frozen and ethanol fixed. For the latter, rat brains were frozen on dry ice and stored at -80°C. Cryostate sections were fixed for 1 min in 70% ethanol.

All sections were dehydrated in three changes of ethanol and twice in xylene. After dehydration, about 50 cells were captured from the sections with the use of a PALM® Microlaser System and the PALM® Laser Microdissection & Pressure Catapulting- (LMPC-) technology (P.A.L.M. Microlaser Technologies AG, Bernried, Germany); these cells were visually identified as dentate granule cells, based on the shape and location of their somata (see below). Collection was confined to the somata, while surrounding tissue was avoided (see Fig 1A). After collection, each sample consisting of 50 cells was transferred to an Eppendorf tube containing 1 μl of oil. Overall, 30 samples of 50 cells were collected in paraformaldehyde fixed sections and 12 samples in ethanol fixed sections.

Fresh dentate granule cells were collected from alive hippocampal slices (400 μm) prepared from 6 week old rats (Karst and Joels, 2001). Under visual guidance, granule cells from the dentate gyrus were identified based on the shape and location of the soma. The soma was then approached with a patch clamp electrode. After establishing a giga-seal, the membrane under the electrode was disrupted and neurons were recorded in the whole cell recording mode. A standard protocol was run to evoke voltage-dependent calcium currents, which served to further identify the recorded cells as dentate granule cells (Karst and Joels, 2001). Based on these physiological properties, we confirmed that the visually selected cells were indeed in all cases granule neurons. After recording the content of the cell, including the nucleus, was aspirated into the pipette (see Fig 1B).

*RNA extraction*

To optimize a procedure for extracting RNA from fixed cells three different protocols
were tested: 1) An amount of 9 µl DEPC treated water or 2) of 1x TE buffer was added to an Eppendorf tube containing 1 µl of LDM collected cells. Subsequently, 50 µl of phenol and of chloroform was added; this mixture was briefly vortexed, and spun down at 14000 rpm for 2 mins. The upper phase was transferred and precipitated in 250 µl cold ethanol containing 10 µl NaAc and 1 µl / 50 ng tRNA. The precipitated pellet was suspended into 10 µl water. 3) An amount of 9 µl of 1x RT buffer (for composition see Qin et al., 2003) was added to an Eppendorf tube containing the LDM collected cells; this was briefly vortexed, spun down at 14000 rpm for 2 mins. Next, the mixture was heated at 50°C for 5 mins, 1 µg of Proteinase K was added and heated at 53°C for 1 hour. Subsequently, 0.5 µg of linear polyacrylamide (LPA) and 200 µl Trizol reagent (Invitrogen, Cat 15596) were added and mixed. Next, 24 µl of chloroform was added, the mixture was briskly vortexed for 30 seconds and spun down. The aqueous phase was transferred to an Eppendorf tube containing 72 µl isopropanol and briefly vortexed. Ethanol precipitation was done in the same way as described above. The pellet was dissolved in 10 µl water for subsequent RNA amplification.

In fresh material, the cell content of single granule cells was aspirated into an Eppendorf tube containing 20U RNase inhibitor (Karten et al., 1999). Next, water was added up to 10 µl for subsequent RNA amplification.

**Figure 1A:**

![Figure 1A](image)

*Figure 1:* Collection of RNA from dentate cells was performed with laser dissection microscopy (LMPC) or through a patch clamp electrode.

A. The left and middle panel show microscopical overviews of dehydrated paraffin embedded, paraformaldehyde fixed hippocampal sections. Small groups of about 50 cells were outlined in the granule cells layers. Detailed inset shows how granule cell somata were removed to collect RNA with LMPC. The far right panel shows a frozen, ethanol fixed section. This section was Nissl counterstained and coverslipped, to check for complete cell dissection.

B. After formation of a gigaseal with a patch clamp recording electrode on a dentate granule cell identified under visual control in an alive hippocampal slice, calcium currents were recorded in the whole cell recording mode. After recording, the electrode with the soma attached was elevated under visual control. The electrode tip and attached soma were then collected for
Both the RNA extracted from LDM captured fixed cells as well as from fresh cells was amplified through a T7 promoter mediated in vitro transcription method, based on protocols described by Eberwine et al. (1992), except for small details mentioned below. In brief, 10 μl of total RNA extracted from either fixed or fresh cells was subjected to an RT reaction at 39 °C for 2 hours (20 μl: 1xRT buffer, 8 mM DTT, 10U RNAse, 250U Seikagaku). Then, the first strand cDNA was purified with phenol:choloroform and ethanol precipitated. Second strand cDNA was synthetized in a 10 μl reaction at 14 °C for 8 hours, and 37°C for 1 hour (1xSSB buffer, 0.2 μM dNTPs, 8 mM DTT, T4 polymerase 2U, Klenow 2U). After phenol:chloroform extraction and ethanol precipitation, double stranded cDNA was treated with S1 nuclease in a 20 μl reaction volume at 37 °C for 7 mins (1xS1 buffer, S1 10U), followed by a filling-in reaction in a 10 μl reaction volume at 37°C for 2 hours (1x FKI, 8 mM DTT, 0.2 μM dNTPs, 2U T4 polymerase). Next, the cDNA was purified by phenol:chloroform, precipitated with ethanol and the pellet was dissolved in 10 μl for a subsequent first round RNA amplification, which was done in a 25 μl reaction (1x aRNA buffer, 8 mM DTT, 0.2 μM ATP, 0.2 μM UTP, 0.2 μM GTP, 0.2 nM CTP, 4U RNAse, 2.5 μl ^32P CTP and 500U T7 polymerase) at 37°C for 8 hours. The amplified RNA was extracted, precipitated and served as starting material for a 2nd round of RNA amplification. The 2nd round amplification was done in the same way as described above. Following second round amplification, the RNA was subjected to slot-blot hybridization.

Slot-blot hybridization

For examination of gene expression 23 cDNA clones as well as a hybridization control, i.e. the empty vector of pBlueScript, were used (see Fig 2D). Of these only 12 yielded a relatively strong signal (see below), whereas very low hybridization signals were observed for the rest, possibly due to their low expression in dentate granule cells. For the present study, comparison between signals from fresh and fixed cells were
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confined to the 12 cDNA clones yielding clear hybridization signals (see also below). These included: i) the calcium channel α1C and α1A subunit and the calcium-calmodulin kinase II; ii) the NMDA receptor subunits NR1, NR2B and NR2C; iii) the pro-apoptotic markers Bax and Caspase 3 (Cpp32) and the anti-apoptotic marker Bcl-2; and iv) three high-abundant proteins, i.e. heat-shock protein 90 (hsp90), cAMP response element binding protein (CREB) and cyclo-oxygenase 2 (COX). The expression of these genes has also been investigated in earlier studies focusing on neurotransmission, cell death and electrophysiological responses (Karten et al., 1999; Karst et al., 2002; Qin et al., 2003).

In detail, each plasmid containing the gene of interest was maxiprep-prepared (Promega, the Netherlands) and 3 μg was linearized with a specific restriction enzyme, heat denatured at 90°C for 5 mins, and then immobilized to a Hybond-N membrane with a blot apparatus (BioRad, USA). The blots were cross-linked and air-dried for subsequent pre-hybridization at 40°C for 6 hours (50% formamid, 5X SSC, %x Denhardt, 0.5% SDS, 100 μg salmon sperm, 1 mM NAPPi). The radioactively labeled, two round linearly amplified RNA was denatured at 70°C for 10 min prior to being added to the pre-hybridized blots. The hybridization was carried out in the same buffer for 48 hours at 40°C. Following the hybridization, the blots were washed with buffer (2xSSC, 0.1%SDS) for 1 hour, and another wash in buffer (0.5xSSC, 0.5% SDS) for 30 mins (all 40°C). The blots were air dried and exposed to image plates for 48 hours before scanning with an image scanner (Molecular Dynamics, Storm, USA).

Data analysis

Expression levels were determined, based on the radioactive intensity scanned. The grey value for each signal measured was first corrected for non-specific hybridization by subtracting the signal for pBlueScript. Expression level was then normalized against either the total signal per blot or the highly abundant CamKII, a stable signal as described earlier (Karst et al., 2002; Qin et al., 2003). Clones of which the averaged hybridization signal relative to the CamKII signal was less than 5% were excluded from the present analysis. All data were tested for statistical differences with a Student’s t-test, or a non-parametric Mann-Whitney U-test, when a non-parametric test was indicated due to differences in the variance between the two experimental groups. Correlation coefficients were determined with a Pearson test.
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Results

Extraction of mRNA

Isolation of RNA from samples of paraformaldehyde fixed cells was tried in total 6 times, using three different protocols described earlier (see for more details Materials and Methods). In our hands, hybridization signals were obtained from only 20% of the paraformaldehyde fixed samples; the chances on success were not associated with any of the above protocols. Also, we often failed to reproduce hybridization signals, using the exact same sample extraction method again. Importantly though, most blots were empty (Fig. 2A), which could point to a failure to successfully extract RNA from thus collected cells or could be due to ineffective RNA amplification and/or hybridization to the selected cDNA clones. Since RNA that was visually collected from single cells in (alive) hippocampal slices and processed along with the fixed cell samples showed clear hybridization signals (Fig. 2B), we conclude that RNA extraction from the paraformaldehyde fixed cells was apparently often unsuccessful.

In contrast to the cells obtained with the LDM method from paraformaldehyde-fixed tissue, LDM collected cells from frozen and ethanol fixed tissue showed reliable and reproducible hybridization signals (Fig. 2C). From all of the samples from ethanol fixed cells, using RNA extraction protocol #3 described above (see Materials and Methods), hybridization signals of good quality were obtained, allowing analysis.

Figure 2A:

Figure 2: Typical hybridization signals from dentate granule cells (approximately 50 cells per blot) collected in paraformaldehyde-fixed (A) or ethanol fixed tissue (B) or from a single dentate granule cell collected in an (alive) hippocampal slice (C). The identity of the cDNA clones that resulted in an analyzable signal is given below (D). The remaining signals were too low to determine reliably (nd = not determined). Clones were always applied in the order shown in D, including for the examples shown in A-C.
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Figure 2C:

Comparison between expression profiles of fixed and fresh dentate cells

Both the RNA extracted from ethanol fixed cells (about 50 cells per sample) and RNA extracted from single dentate granule cells obtained from (alive) hippocampal slices were taken through the same amplification procedure as well as through identical hybridization protocols. The radioactive signal on blots from fixed samples was consistently lower than the signal on blots from fresh single cells (see examples in Fig. 2B and 2C).

We next examined the effects of ethanol fixation and subsequent LDM based collection of cells on the expression profile. To this end, the relative expression of the selected transcripts in samples from fixed dentate cells was compared with the relative expression levels obtained from fresh cells. Table I shows the relative expression ratios of selected transcripts either against the total expression value of the blots or against the highly abundant and stably expressed CamKII, which was earlier used as a standard (Karst et al., 2002; Qin et al., 2003). With both approaches, none of the ratios showed significant differences between the fixed samples and fresh cells. Thus, after ethanol fixation in combination with LDM collection of cell samples the expression profile turned out to be very similar to that seen in non-fixed fresh single cells.

This was further substantiated by applying linear regression analysis to the relative expression in fixed and fresh cell samples. As shown in Figure 3, a strong, positive correlation was observed between relative expression levels observed in the two groups. This was true both when selected cDNA clones were expressed relative to the total hybridization signal (Fig 3A) and when they were expressed relative to CamKII (Fig 3B). A highly significant correlation ($r^2=0.984$, signal expressed relative to CamKII) was still observed when the remaining 11 transcripts that had been initially excluded, were also included in the analysis, although it should be noted that the hybridization signal for these transcripts was low and less reliable.

Table I: Expression profiles of ethanol fixed dentate cell samples (left; $n=12$ samples, each based on RNA from about 50 cells; numbers represent mean $\pm$ SEM) and non-fixed single granule cells (right, $n=12$ cells). The expression intensity measured on the blot was first corrected by subtracting the signal of the empty vector (pBS). Next, for the 12 transcripts which displayed a relatively strong hybridization signal (see main text), the specific hybridization signal ($\times 100$) was expressed against the total expression value of the blot, i.e. a summation of the signal for
Table 1:

<table>
<thead>
<tr>
<th>Transcript ratio</th>
<th>Relative expression in fixed samples</th>
<th>Relative expression in fresh cells</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1C / total</td>
<td>8.3 ± 0.8</td>
<td>9.2 ± 0.5</td>
<td>0.33</td>
</tr>
<tr>
<td>α1A / total</td>
<td>9.8 ± 0.7</td>
<td>9.4 ± 0.5</td>
<td>0.60</td>
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<tr>
<td>CamKII / total</td>
<td>12.2 ± 0.8</td>
<td>11.7 ± 0.5</td>
<td>0.67</td>
</tr>
<tr>
<td>NR1 / total</td>
<td>10.4 ± 1.0</td>
<td>11.3 ± 0.8</td>
<td>0.48</td>
</tr>
<tr>
<td>NR2B / total</td>
<td>2.4 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>0.37</td>
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<tr>
<td>NR2C / total</td>
<td>1.4 ± 0.2</td>
<td>3.3 ± 1.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Bax / total</td>
<td>9.4 ± 0.5</td>
<td>11.4 ± 1.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Cpp32 / total</td>
<td>11.4 ± 0.5</td>
<td>10.7 ± 1.5</td>
<td>0.70</td>
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<tr>
<td>Bcl2 / total</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.4</td>
<td>0.85</td>
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<tr>
<td>hsp90 / total</td>
<td>1.5 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>0.68</td>
</tr>
<tr>
<td>CREB / total</td>
<td>20.0 ± 2.1</td>
<td>20.2 ± 1.7</td>
<td>0.95</td>
</tr>
<tr>
<td>COX / total</td>
<td>4.0 ± 0.6</td>
<td>3.7 ± 0.3</td>
<td>0.67</td>
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<tr>
<td>α1C / CamKII</td>
<td>76.9 ± 3.2</td>
<td>76.4 ± 4.2</td>
<td>0.92</td>
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<td>α1A / CamKII</td>
<td>83.1 ± 5.9</td>
<td>81.1 ± 4.5</td>
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<td>NR1 / CamKII</td>
<td>87.7 ± 7.4</td>
<td>98.0 ± 7.7</td>
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<td>NR2B / CamKII</td>
<td>21.7 ± 3.5</td>
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<td>NR2C / CamKII</td>
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<td>28.5 ± 10.0</td>
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<td>79.8 ± 5.4</td>
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<td>Cpp32 / CamKII</td>
<td>90.2 ± 4.4</td>
<td>86.2 ± 13.6</td>
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<tr>
<td>Bcl2 / CamKII</td>
<td>8.8 ± 3.2</td>
<td>5.6 ± 1.2</td>
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<td>hsp90 / CamKII</td>
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<td>9.0 ± 1.4</td>
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<td>CREB / CamKII</td>
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<td>176.1 ± 16.7</td>
<td>0.87</td>
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<tr>
<td>COX / CamKII</td>
<td>32.9 ± 5.2</td>
<td>32.6 ± 2.0</td>
<td>0.95</td>
</tr>
</tbody>
</table>

In addition, these relatively strong transcript signals (×100) were expressed relative to the signal for CamKII, which is abundantly and stably expressed in dentate granule cells (Qin et al., 2003). All data were compared with a Student’s t test or Mann-Whitney U-test if the variance between the two groups was significantly different.

Figure 3; Correlation between relative expression of selected cDNA clones in samples from fixed granule cells (average of 12 samples) and in single dentate granule cells obtained from (alive) hippocampal slices (average of 12 cells). In the upper graph, all hybridization signals were expressed relative to the summated signal per blot. In the lower graph, hybridization signals were expressed relative to the signal of CamKII. In both cases, an extremely strong correlation was observed between the averaged expression level in fixed cell samples and the averaged level in fresh cells (upper graph: \( r^2 = 0.974 \); lower graph: \( r^2 = 0.982 \)).
Discussion

RNA from brain tissue, in particular human brain, is often readily available from archival, formalin-fixed and paraffin-embedded material. Since pathophysiological processes are usually confined to specific cell types, it is important not only to collect RNA from fixed tissue but also to confine this to small samples of microscopically identified cells. The recent introduction of the LDM methodology allows a defined sampling of limited amounts of cells, even single cells. The aim of the present study was to generate expression profiles from such small, though homogenous samples of fixed brain cells and to verify the reliability of thus generated profiles by comparing them with expression patterns obtained from single, fresh neurons belonging to the same cell type. Since fresh cells from human brain could not be obtained, the study was performed in rat.

Fixed cell samples collected by LDM methodology differ in several aspects from the fresh single cells. First, the fixation procedure may introduce difficulty in extraction of RNA of sufficient quality from the cells. In agreement with earlier studies using larger tissue samples as starting material, we observed that RNA extraction from formalin-fixed, paraffin-embedded cells was less reliable than extraction from ethanol fixed, frozen cells (Rupp and Locker, 1988; Stanta and Schneider, 1991; Finke et al., 1993; Foss et al., 1994; Karlsen et al., 1994; Serth et al., 2000; Shibutani et al., 2000; Specht et al., 2001; Macabeo-Ong et al., 2002). Ethanol fixation introduces only very little chemical modifications, as it preserves nucleic acids through denaturation; by contrast, formalin causes cross-linking between nucleotides and proteins, often yielding a poor extraction efficiency.

Even when successfully extracted, RNA samples could still have been subject to RNA degradation. RNAses may have been present and active during immersion fixation. However, since the present tissue was perfusion fixed, this possibility is quite remote. In addition to fixation, pre-fixation time, tissue processing conditions, de-paraffinization and LDM collection are all conditions expected to introduce RNA degradation. Also, DNase treatment with subsequent phenol/chloroform purification may further reduce the final RNA yield considerably (Srinivasan et al., 2002). This can become a serious problem if only small amounts of RNA are used as starting material. Interestingly, despite the fact that the hybridization signals on blots incubated with fixed cells were derived from about 50 cells, they were consistently weaker than the signals obtained from single fresh dentate cells, suggesting that also ethanol fixation may have induced some RNA loss. It should be noted, though, that the present method is not quantitative (Eberwine et al., 1992), so that absolute intensity of signals should be carefully interpreted.
Apart from degradation, RNA could also undergo modifications that interfere with the amplification process. For instance, a high frequency of sequence alterations has been reported with formalin compared to ethanol fixation (Williams et al., 1999). Also, as adenine is known to be most susceptible to formaldehyde-methylene bridges (McGhee and von Hippel, 1977), the poly(A) tail of fixed mRNA may have been modified by formalin. Hence, oligo (dT) would not anneal very well to the poly(A) tail, preventing proper reverse transcription for cDNA synthesis. For cellular RNA, the methylol addition may also hamper reverse transcription and cDNA synthesis, although this can partly be reversed by heating. Finally, sectioning of the tissue is likely to affect or destroy the cellular integrity, since the size of the dentate granule somata often exceeds the thickness (8 μm) of the sections.

Despite all these possible limitations, the present data shows that the relative expression ratios in samples from fixed cells and single fresh cells were highly comparable. We confined our analysis to those transcripts that yielded a strong hybridization signal, since this limits the variance between observations within the same treatment group to 5-10% of the mean value. Transcripts with a low intensity are much more variable (Qin et al., 2003). Yet, even when these lower intensity signals were included in the analysis, correlation between signals from fixed and fresh cells remained extremely high.

In conclusion, the present study shows that RNA was successfully extracted from ethanol fixed sections and yielded expression profiles that were highly comparable to those of non-fixed, single granule cells. In our hands, and with the present protocols tested, RNA could not be reliably extracted from paraformaldehyde fixed, paraffin embedded tissue. The present approach provides a powerful tool to investigate expression profiles from small amounts of histochemically identified neurons especially if fresh tissue of life slices is not available.
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frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol, 1999; 155: 1467-71.

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