Transient hippocampal down-regulation of Kv1.1 subunit mRNA during associative learning in rats

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Voltage-gated K⁺ (Kv) channels play an important role in regulating neuron excitability. Although their primary function is to stabilize membrane potential, they can also regulate cell firing and information transfer rates to effectively prevent hyperexcitability in neuronal networks. The Kv1 channel, one of four subfamilies identified (Kv1–Kv4), consists of four primary subunits, which can be further classified as cloned subunits. At least five α subunits (Kv1.1–Kv4, Kv1.6) are highly and diversely expressed in the limbic system (Kues and Wunder 1992; Sheng et al. 1993, 1994; Wang et al. 1994; Veh et al. 1995; Rhodes et al. 1997; Monaghan et al. 2001). A large variety of Kv1 channel blockers are available, making it possible to discriminate between the various K⁺ currents. Unfortunately, these blockers recognize several Kv1 subunits and not one subunit only (Stühmer et al. 1989; Grissmer et al. 1994; for review, see Coetzee et al. 1999; Harvey 2001). However, they facilitate the investigation of Kv1 channel physiology (for review, see Robertson 1997; Song 2002). The pharmacological blockage of Kv1 channels increases the duration of action potential, and as a result, voltage-gated Ca²⁺ channels remain open for a much longer period of time, thereby increasing the amount of neurotransmitter released from presynaptic terminals (Meir et al. 1999; Cunningham and Jones 2001; Monaghan et al. 2001). Moreover, inactivation of presynaptic Kv1 channels in vitro can significantly affect activity-dependent mechanisms involved in spike-broadening, and thus powerfully regulate synaptic efficacy in hippocampal slices and in the medial nucleus of the trapezoid body slices (Geiger and Jonas 2000; Dodson et al. 2002; Dodson and Forsythe 2004). The heteromer channels containing Kv1.1/Kv1.2 and Kv1.1/Kv1.6 are involved in such regulatory actions (Dodson et al. 2002).

Enhanced synaptic transmission and a reduction in K⁺ conductance can increase behavioral efficacy on tasks requiring learning and memory processes. Indeed, a correlation has been found in *Aplysia* between reduced K⁺ currents and learning (Alkon 1984, 1999). Furthermore, in *Drosophila*, a genetic shaker mutation of Shaker A-type K channel inactivation produces learning impairment in olfactory conditioning (Cowen and Siegel 1986). In mammals, conditioning can cause significant reduction of K⁺ currents (Sanchez-Andres and Alkon 1991; Schreurs et al. 1998). A reversible antisense inhibition of Kv1.1 channel expression impaired associative memory in rodents (Meir et al. 1997; Gratacos et al. 1998). Moreover, Kv1.1-deficient mice suffered an impaired learning in a water-maze task. The loss of Kv1.1, an auxiliary subunit that confers fast inactivation on otherwise noninactivating Kv1.1 channels, leads to a reduction of frequency-dependent spike broadening (Giester et al. 1998, 2001). A transient increase in paired pulse facilitation in the piriform cortex evoked by afferent fiber stimulation in vitro is related to enhanced learning capabilities following odor-discrimination training. This facilitation was followed by a persistent reduction in K⁺ currents that returned to baseline values after 5 d (Saar et al. 1999). The inhibition of Kv1 activity by 4-diaminopyridine was found to reduce K⁺ currents and improve memory task performance in old rats (Barnes et al. 1989). In a recent study, Kourrich et al. (2001) showed that kaliotoxin, a selective blocker of Kv1.1 and Kv1.3 channels, facilitated learning processes, but not long-term storage of an odor-reward association in an olfactory task. In concordance, these observations support the idea that Kv1 channels, in particular, one containing the Kv1.1 subunit, essentially participate in the regulation of neurotransmitter release, and consequently synaptic efficiency during learning and memory processes. Moreover, the physiological modulation of Kv1.1 channels seems to be orchestrated, in principal, by synergetic actions of protein kinases (Drain et al. 1994; Boland and Jackson 1999; Manganas and Trimmer 2000; Winklhofer et al. 2003). Relatively little is known, however, about physiological regulation of Kv1 channel genes during these two processes.

The aim of the present study was to analyze possible variations in Kv1.1 subunit expression during associative learning. We used an odor-discrimination task in rats because (1) kaliotoxin...
improves learning performance in this task (Kourrich et al. 2001), and (2) the expression of the activity-regulated immediately early gene c-fos (which is used as an “activity marker” and a “marker of changes in gene expression”) changes during a two odor-discrimination learning task. Dorsal hippocampal fields are differentially activated during specific stages of learning (Hess et al. 1995; Gall et al. 1998). Although dentate gyrus labeling remained stable throughout learning, c-fos hybridization levels were enhanced in areas CA1 and CA3 fields, and were highest in CA3 during the initial learning of an odor pair when compared with overtrained rats (Hess et al. 1995; Gall et al. 1998). We show here that the level of Kv1.1 mRNA transiently decreases throughout associative learning, and that significant changes in levels occur in the ventral hippocampus prior to changes in the dorsal hippocampus. Furthermore, we also showed that Kv1.1 mRNA expression is positively correlated with learning on a memory task.

Results

Acquisition of the odor-reward association

During the associative olfactory task, conditioned animals improved across sessions prior to their sacrifice. The comparison of percent correct responses indicated a significant difference between the second and third sessions (ANOVA, \(F_{1,21} = 4.759, P < 0.05\)) (Fig. 1A). A significant latency difference between positive stimulus (S+) and negative stimulus (S−) was observed in session 3 as revealed by ANOVA (\(F_{1,34} = 6.805, P < 0.05\), but not in session 2 (ANOVA, \(F_{1,34} = 0.404, \text{NS}\)). This significant discrimination was due to a decrease in the time taken to respond to positive stimulus, and an increase in response time to negative stimulus (Fig. 1B). The cumulative time decreased over sessions (Fig. 1C), indicating successful procedural learning aspects of the associative task.

![Figure 1](https://www.learnmem.org)

**Figure 1.** Odor-reward training. Mean performance obtained across three 60-trial sessions. The intertrial interval was 15 sec. (A) Correct response rate ± S.E.M. (in percent); (B) Latencies ± S.E.M. (in seconds). S+ and S− latencies were recorded for the positive and negative odor stimulus, respectively; (C) cumulative time ± S.E.M. (in seconds). Note the significant discrimination of the two odors in the third session. (*) \(P < 0.05\) (session 2, \(n = 18\); session 3, \(n = 5\)).

Kv1.1 in situ hybridization

Animals from the three experimental groups (naive, pseudoconditioned, and conditioned) were decapitated at different times as follows: 1 h after the second session, 4 h after the second session, and 1 h after the third session. In total, two in situ hybridization (ISH) experiments were performed, the first to compare Kv1.1 expression in different groups at a given time (comparison of mRNA levels between the groups) and the second to analyze the temporal pattern (time course) of Kv1.1 mRNA expression for a given experimental group (temporal analysis). The statistical comparisons were performed from brain sections hybridized in the same experiment and autoradiographed using an identical film sheet.

Figure 2 shows examples of the specific ISH signal patterns observed in the parietal and occipital cortices, ventral posterior-medial thalamic nucleus, geniculate nuclei, red nucleus, and hippocampal formation. In the hippocampus, Kv1.1 mRNA expression was weak in the pyramidal layer of the CA1 field, whereas the signal was intense in the pyramidal layer of the CA3 field and in the granular layer of the dentate gyrus. In contrast, the olfactory bulb contained a weak Kv1.1 mRNA amount in the internal granular layer (data not shown).

Comparison of Kv1.1 mRNA levels between the groups

In the examination of olfactory pathways, an analysis was performed on regions believed to be necessary for the learning and memory of olfactory associative tasks (olfactory bulb, Ammon’s horn, and dentate gyrus) (Chaillan et al. 1999; Truchet et al. 2002), as well as in the three animal groups (naive group, pseudoconditioned group, and conditioned group). The Kv1.1 mRNA level was analyzed in the olfactory bulb and in three structures of the dorsal and ventral hippocampus (dentate gyrus, CA3 and CA1) of rats sacrificed at three stages of learning, i.e., 1 h after session 2, 4 h after the second session, and 1 h after session 3. Kv1.1 ISH labeling was not altered in the internal granular layer of the olfactory bulb, regardless of the task session or sacrifice time (ANOVA, \(F_{2,35} = 2.876, \text{NS}\)) (Table 1). In contrast, the mRNA level varied in the hippocampal formation (see below).

**Table 1.** Kv1.1 mRNA levels in the anterior olfactory nucleus

<table>
<thead>
<tr>
<th>Session</th>
<th>1 h</th>
<th>4 h</th>
<th>1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>100.0 ± 2.3</td>
<td>112.3 ± 6.1</td>
<td>109.4 ± 3.8</td>
</tr>
<tr>
<td>Pseudoconditioned</td>
<td>100.0 ± 3.1</td>
<td>111.7 ± 4.3</td>
<td>125.9 ± 4.9</td>
</tr>
<tr>
<td>Conditioned</td>
<td>100.0 ± 10.3</td>
<td>108.9 ± 11.3</td>
<td>112.2 ± 5.6</td>
</tr>
</tbody>
</table>

In the examination of olfactory pathways, an analysis was performed on regions believed to be necessary for the learning and memory of olfactory associative tasks (olfactory bulb, Ammon’s horn, and dentate gyrus) (Chaillan et al. 1999; Truchet et al. 2002), as well as in the three animal groups (naive group, pseudoconditioned group, and conditioned group). The Kv1.1 mRNA level was analyzed in the olfactory bulb and in three structures of the dorsal and ventral hippocampus (dentate gyrus, CA3 and CA1) of rats sacrificed at three stages of learning, i.e., 1 h after session 2, 4 h after the second session, and 1 h after session 3. Kv1.1 ISH labeling was not altered in the internal granular layer of the olfactory bulb, regardless of the task session or sacrifice time (ANOVA, \(F_{2,35} = 2.876, \text{NS}\)) (Table 1). In contrast, the mRNA level varied in the hippocampal formation (see below).

**Stage: 1 h after the session 2 (Fig. 3)**

Statistical analysis of data from the ventral hippocampus indicated a significant decrease of ISH labeling for each structure analyzed, that is, the conditioned group in comparison with the pseudoconditioned and naive groups (ANOVA, \(F_{2,35} = 3.802, P < 0.05\)). In both the dentate gyrus and CA3 field, Kv1.1 mRNA expression was significantly decreased in the conditioned group compared with that of the pseudoconditioned group (−40%, \(P = 0.049\) and −35%, \(P = 0.047\), respectively). Messenger RNA levels for the conditioned group also decreased (−37%, −30% respectively) in comparison with that of the naive group, but this decrease was not significant. Furthermore, no variation between the pseudoconditioned and naive groups was found. In the CA1 field, however, the mRNA levels were significantly lower in the conditioned than in both the pseudoconditioned (−44%, Scheffé, \(P = 0.044\)) and the naive (−47%, Scheffé, \(P = 0.018\)) groups.
In the dorsal hippocampus, statistical analysis indicated no significant variation of Kv1.1 mRNA levels between each of the groups for the three analyzed structures \( F(2,35) = 1.506, \text{NS} \).

Stage: 4 h after the session 2 (Fig. 4)
In the ventral hippocampus, a difference in mRNA expression was found in the CA1 field (ANOVA, \( F(2,33) = 3.687, P < 0.05 \)) with a significant decrease in the conditioned group compared only with that of the naive group \((-31\%, \text{Scheffé, } P = 0.047\)).

In contrast, in the dorsal hippocampus, Kv1.1 mRNA expression showed significant variations in the three regions examined (ANOVA, \( F(2,33) = 3.459, P < 0.05 \)). The Kv1.1 mRNA level decreased between the conditioned and the naive groups for the dentate gyrus, CA3, and CA1 field \((-38\%, -28\%, \text{and } -37\%, \text{Scheffé, } P = 0.018, P = 0.042, \text{and } P = 0.046, \text{respectively} \)). ISH labeling in the dorsal hippocampus for the conditioned group was weaker than that of the pseudoconditioned group, (CA1, -26%, CA3, -11%, and Dentate gyrus, -40%), but this difference was not significant.

Stage: 1 h after the session 3 (Fig. 5)
In the ventral hippocampus, Kv1.1 mRNA levels in the dentate gyrus and CA3 field were similar between the three groups (ANOVA, \( F(2,31) = 2.668, \text{NS} \)). In contrast, a significant change in the mRNA level was observed in the CA1 field (ANOVA, \( F(2,29) = 3.554, P < 0.05 \)). The Kv1.1 mRNA expression in the conditioned group CA1 field was significantly increased (+48%) when compared with that of the naive group (Scheffé, \( P = 0.048 \)), but not to that of the pseudoconditioned group.

In the dorsal hippocampus, ANOVA indicated a significant difference in the dentate gyrus (\( F(2,31) = 4.438, P < 0.05 \)), but not in the CA1 and CA3 fields (\( F(2,31) = 0.919, \text{NS} \)). The Kv1.1 mRNA level in the dentate gyrus of the pseudoconditioned group was decreased in comparison to that of the naive group \((-35\%, \text{Scheffé, } P = 0.025\)). However, no relative variation was found in the conditioned group.

Temporal analysis (Fig. 6)
The aim of this analysis was to study potential variations in Kv1.1 mRNA levels throughout a learning and memory task in a given experimental group with a given brain structure. All compared data was obtained from autoradiograms on the same film sheet.

Throughout the odor-discrimination task procedure, Kv1.1 mRNA amounts increased in the olfactory bulb, especially in the pseudoconditioned group (ANOVA, \( F(2,15) = 6.931, P < 0.01 \)). The Scheffé analysis indicated an increase 4 h after session 2, which remained throughout session 3, compared with 1 h after session 2 (+22%, \( P \leq 0.02 \)). No variations of mRNA levels were statistically observed for the conditioned group. No labeling variation was found in the dorsal CA1 field of the hippocampus for both groups (ANOVA, \( F(2,15) = 2.312, \text{NS} \)). In the ventral CA1 field, the Kv1.1 mRNA level significantly increased in the pseudoconditioned group, whereas no differences were observed in the conditioned group (ANOVA, \( F(2,15) = 4.340, P < 0.05 \); \( F(2,15) = 1.802, \text{NS} \), respectively). ISH level increase in the ventral CA1 field was
Learning and memory processes during an odor-reward associative task led to a decrease in Kv1.1 subunit mRNA in the hippocampus. More precisely, the results suggest that the decrease in mRNA expression occurs when rats begin to learn odor-reward association, and is transient because no mRNA level differences between conditioned and pseudoconditioned rats were observed once learning stabilized in session 3. Moreover, the Kv1.1 mRNA level was positively correlated with learning.

The distribution of Kv1.1 subunit mRNA in the experimental group was consistent with previous studies showing a high level in the CA3 field and dentate gyrus, and a low level in the CA1 field, olfactory bulb, and neocortex (Kves and Wunder, 1992; Wang et al. 1994; Hallows and Tempel 1998). Subcellular localization of Kv1.1 protein in the hippocampal formation was found to be localized primarily on axon and terminal regions of mossy fiber afferents, Schaffer collaterals, and in the entorhinal cortex (Monaghan et al. 2001). However, dendritic Kv1.1 subunit was observed in some brain structures, such as the apical dendrites of pyramidal cells throughout the neocortex (Wang et al. 1994; Veh et al. 1995).

Behavioral performance on olfactory associative discrimination was similar to our previous reports (Fournier et al. 2001; Kourrich et al. 2001). During session 2, the animals did not learn the two-odor significance, as they rapidly responded to both the S+ and S− stimuli. In conditioned animals, Kv1.1 mRNA levels were decreased in both the ventral and dorsal hippocampus at 1- and 4-h intervals following session 2, compared with pseudoconditioned and naive animals, respectively (Table 2). Following session 2, however, no Kv1.1 mRNA level differences were detected between pseudoconditioned rats exposed to the same stimulus level compared with water restricted naive rats. This suggests that exposure to odors, water, and light pulses was not sufficient to generate the observed decreases in Kv1.1 mRNA, but rather reflects pure learning processes. Because the stimuli were not significant for pseudoconditioned rats, the decrease in Kv1.1 mRNA suggests modifications of neuronal excitability balance during training.

Throughout session 3, the animals showed a preference for S+ stimulus, and did not respond to S− stimulus; at the same time, the ISH level did not vary during odor-discrimination learning (ANOVA, F1.5 = 2.457, NS). In contrast, if no variation was found in the dorsal part of the dentate gyrus, a significant decrease in Kv1.1 mRNA levels were detected in the ventral part 4 h after session 2 in the conditioned group (ANOVA, F1.5 = 11.067, P < 0.001). Furthermore, this observed decrease persisted throughout session 3 (−22% and −22%, Scheffé, P = 0.013 and P = 0.001, respectively).

**Analysis of Kv1.1 mRNA level in relation to behavior**

To examine potential relationships between behavior and Kv1.1 mRNA levels during the olfactory discrimination task, we performed linear regression at specific stages of learning, and in particular, where statistical differences in ISH labeling between rat groups were observed (i.e., 1 h after session 2 for the ventral hippocampus). At 1 h following session 2, the regression line between S− stimulus performance and Kv1.1 mRNA levels of the three ventral hippocampus structures was significant (Fig. 7, F1.5 ≥ 6.996, P < 0.05). Moreover, correct response for S− stimulus was also correlated with the mRNA level in the dentate gyrus, CA3, and CA1 fields of the ventral hippocampus (F1.5 ≥ 6.617, P < 0.05). In contrast, where there was a zero slope, no significant correlation was found for S+ stimulus, global correct response, and cumulative time (Fig. 7) regardless of structures studied (F1.5 ≤ 1.521, NS). Odor discrimination was evaluated by an S+ and S− latency analysis and the latency difference was correlated significantly with mRNA levels in the dentate gyrus and CA3 field of the ventral hippocampus (F1.5 ≥ 6.901, P < 0.05), but not in CA1 field, although the line slope showed a positive relationship between latency differences and mRNA levels (Fig. 7). No significant regressions were found between the other learning stages and brain structures (data not shown).

**Discussion**

Learning and memory processes during an odor-reward associative task led to a decrease in Kv1.1 subunit mRNA in the hippocampus. More precisely, the results suggest that the decrease in mRNA expression occurs when rats begin to learn odor-reward association, and is transient because no mRNA level differences between conditioned and pseudoconditioned rats were observed once learning stabilized in session 3. Moreover, the Kv1.1 mRNA level was positively correlated with learning.

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the odors during session 3. Gene expression regulation can occur at transcription, translation, or post-translational factor levels. cAMP level modulation may be one possible explanation for Kv1.1 mRNA down-regulation. Indeed, when cAMP levels were elevated in C6 glioma cells, Kv1.1 protein and the peak current amplitude of a delayed rectifier type of K+ current were decreased. These were preceded by strong, rapid decreases in steady-state levels of Kv1.1 RNA (t1/2 < 3 h) resulting from a post-transcriptional destabilization of the transcript (Bosma et al. 1999; Mori et al. 1993; Allen et al. 1998; Matthias et al. 2002). Learning to associate a stimulus to a procedural behavior process (go or not go) for (or not) rat reinforcement may be related to receptor stimulation by neurotransmitter release in the hippocampus, and as a consequence, increase the cAMP level and subsequently decrease the Kv1.1 mRNA amount.

The contribution of Kv1.1 subunits to synaptic efficiency during information acquisition was reinforced by actual knowledge of the involvement of Kv1.1 channels in fine-tuning network excitability and the modulation of transmitter release. The Kv1.1 subunit is principally located in presynaptic sites, in the initial segment of the trapezoid body medial nucleus (MNTB) (Dodson et al. 2002), and in several CNS nerve terminals within transition zones between axons and the synaptic terminals (Wang et al. 1994; Veh et al. 1995; Rhodes et al. 1997; Monaghan et al. 2001). Kv1.1 subunits co-assembled with Kv1.2 subunits constitute Kv1 heteromer channels responsible for low-threshold voltage-gated potassium currents (I_{L,T}) sensitive to low concentrations of dendrotoxin-I (Dodson and Forsythe 2004). The physiological function of the I_{L,T} current, activated around the threshold for action potential regeneration is to suppress hyperexcitability and reduce aberrant action potential generation. This is achieved by maintaining the membrane potential below threshold during small depolarizations, and by bringing the membrane back below threshold after single action potential. Dendrotoxin-K, which blocks channels containing Kv1.1, blocks

Regression analysis between Kv1.1 mRNA levels and behavioral learning revealed a positive correlation, suggesting that decreases in mRNA levels occur before the early two-odor learning process, as low differences between S+ and S − latencies related to low mRNA levels, and high-latency differences to high mRNA levels. This down-regulation of Kv1.1 mRNA was transient and disappeared when the rats successfully discriminated between the naive (n = 6), pseudoconditioned, (n = 5), and conditioned (n = 5) groups.

Figure 5. Kv1.1 subunit mRNA levels, measured 1 h after session 3, for ventral and dorsal hippocampus regions. Data expressed as mean ± S.E.M. Statistical significance: (*) P < 0.05 in ANOVA comparing the naive (n = 6), pseudoconditioned, (n = 5), and conditioned (n = 5) groups.


data

Figure 6. Temporal analysis, Kv1.1 subunit mRNA levels for olfactory bulb and dorsal and ventral hippocampus regions in the pseudoconditioned (A) and conditioned groups (B). Data expressed as mean ± S.E.M. Statistical significance: (*) P < 0.05 in ANOVA comparing the different stages of learning, 1 h after session 2 (n = 6 and 7 for pseudoconditioned and conditioned group, respectively), 4 h after session 2 (n = 6 and 6), and 1 h after session 3 (n = 5 and 5).
the $I_{\text{A}}$ current in MNTB neurons and generates multiple action potentials (Dodson et al. 2002). Moreover, Kv1.1 subunits coassembled with Kv1.4 or Kv1β subunits were also responsible for a transient current (A-type current) that permitted short-term spike threshold modulation, as well as action potential modulation duration. Cumulative inactivation of the A-type current using a sequence of stimuli results in presynaptic action potential broadening, and hence, transmitter release potentiation (in mossy fiber boutons in rat hippocampal slices [Geiger and Jonas 2000]; in neurohypophysial terminals [Jackson et al. 1991]). In the instance where mRNA level decrease reflects repressed protein synthesis, the Kv1.1 mRNA decrease, observed during the olfactory associative discrimination task may act to diminish threshold impact, voltage-gated potassium, and/or A type currents, and thus, contribute to the dynamic synaptic strength regulation during memory processing. As observed in the present study, Kv1.1 and Kv1.3 channel activity was inhibited by kaltoxin in vivo, (which may have pharmacologically reinforced reduced Kv1.1 mRNA effects) during the olfactory discrimination task, where KTX-treated rats had better information acquisition performances (Kourrich et al. 2001).

Intracerebroventricular injections of Kv1.1 DNA antisense oligonucleotides reduce Kv1.1 mRNA (around 30%) and induce a consistent spike waveform broadening in dentate granular cells in hippocampal slices. This was mainly attributed to a late rectifying K+ current decrease in consequence, a prolonged repolarization phase and an after-hyperpolarization suppression (Meiri et al. 1997). Antisense inhibition of Kv1.1 impaired learning and memory for spatial and nonspatial contexts without affecting long term potentiation (Meiri et al. 1997; Gratacos et al. 1998). The induced reduction degree of Kv1.1 mRNA was similar to that found in the present study. However, the antisense knockdown was performed throughout training and affected the entire hippocampus, whereas a transient Kv1.1 mRNA reduction was found in only a few hippocampal fields. This difference might explain the comparison between the controls and the antisense treatment memory impairment observed. In mice lacking the Kv1.1 subunit, recordings from CA3 pyramidal cells showed normal action potential properties; however, orthodromic or antidromic stimulation could evoke synaptically driven bursts of action potential contrary to control, and suggests that the lack of Kv1.1 subunits can lead to synaptic hyperexcitability (Smart et al. 1998; Lopantsev et al. 2003). MNTB neurons recorded from mutant Kv1.1 knockout mice were found to be more excitable than those from wild-type controls. This may reflect a low voltage-activated K+ current (half the amplitude of wild types), an increased firing frequency, or a reduced threshold for action potential generation (Brew et al. 2003). Taken together, the results of the present study suggest that Kv1 channels containing Kv1.1 subunits contribute to synaptic excitability modulation during learning and memory processes, and that this involvement is critical to the ventral hippocampus at the early learning stages.

Figure 7. Relationships between the learning and Kv1.1 subunit mRNA levels measured 1 h after session 2 for the conditioned group in ventral hippocampus fields. Latency difference corresponds to differences between the S+ stimulus latency and the S− stimulus latency. Correlations for Kv1.1 expression and S− latency could be established. Simple-linear regression analysis. Statistical significance: $P < 0.05$.

### Materials and Methods

#### Animals

Before olfactory associative learning and in situ hybridization, 52 male Sprague Dawley rats (280–300 g) (Charles River Company) were housed individually and given food and water ad libitum at a constant temperature (22°C) under a 12 h light/12 h dark cycle (lights on at 7:00 a.m.). Each animal was handled (10 min) for 1 d before the behavioral experiment. These rats were divided into three experimental groups, one group of naive control animals that remained in the animal room, but were subjected to a water deprivation protocol ($n = 18$), a second group of pseudoconditioned rats ($n = 17$), and a third group of conditioned rats ($n = 17$). In the olfactory associative task, over a 2-d period, the pseudoconditioned and conditioned rats were handled again and put into experimental cages for experimental environment familiarization. The rats were deprived of water for 48 h prior to the first training session. On consecutive days, the rats were given water ad libitum for 30 min per day at 5:30 p.m.

#### Olfactory associative task

**Apparatus**

Discrimination training was conducted in a behavioral apparatus similar in design to Roman et al. (1993) and Kourrich et al. (2001). In brief, the apparatus was a rectangular box made of wire mesh (30 × 30 × 50 cm). A conical odor port (1.5 cm in diameter, 0.5 cm above the floor) was drilled horizontally through a
Dorsal hippocampus

Each of the odors had to be associated with a specific reward; one odor (S+) flask was paired with a “positive” odor (5+) reward and the other (negative) odor was paired with a reward. Correct responses were “go” for the positive odor and “no go” for the negative odor. Incorrect responses were “go” for the negative odor and “no go” for the positive odor. The number of correct responses to both positive and negative odors was expressed as a percentage of the total number of odor presentations. Latencies for positive (S+) and negative (S−) odors were recorded, representing the time elapsed between the beginning of a trial and its end when the rat responded to the odor. If a rat did not respond, a latency of 10 sec was scored. The percentage of correct responses and the latency were used to assess discriminative performance. In addition, because the subject could delay the trials by responding during the intertrial interval, the cumulative time was considered. The cumulative time was the number of seconds that exceeded the fixed (15-sec intertrial) interval divided by the number of intertrial intervals in the experiment.

Odor-reward training for pseudoconditioned animals

Pseudoconditioned animals were prepared in the same way as conditioned animals. However, from sessions 1 to 3, they experienced explicitly unpaired presentations of a positive odor, a negative odor, water, and light flashes. For each session, the odor stimuli was presented 30 times, and the number of water and light-flash presentations corresponded to the mean number of those accumulated during each session of the conditioned rats. Each of the four stimuli was presented in a quasi-random fashion. The duration of sessions 1–3 for pseudoconditioned animals was the mean duration of session for conditioned animals.

Tissue preparation

At the end of each training session, all rats were replaced in the animal room. Some of them from the three experimental groups (naive controls, pseudoconditioned, and conditioned) were anesthetized with pentobarbital sodic (6%, Sanofi sante) and killed at different times, 1 h after the second session, 4 h after the second session, and 1 h after the third session. These times were chosen when the rats had not learned the two-odor significance following the second session, but successfully discriminated between them after the third session. Rats were sacrificed 1 h after the second session when kalotoxin (a toxin that inhibits the Kv1.1 α-subunit) was found to improve associative learning following the same odor-reward training protocol (Kourrich et al. 2001). In addition, a 4-h time period was chosen, as it corresponded to the late stage of long-term potentiation, a model for memory processes related to protein synthesis and gene transcription (Bliss and Collingridge 1993; Izquierdo and Medina 1997). Brains were immediately removed, frozen on powdered dry ice, and stored (−80°C). Coronal sections (12 µm) were cut in a cryostat at −20°C, thaw mounted on gelatin-coated slides, and stored at −80°C.

Oligonucleotide probes

An oligodeoxynucleotide probe for the rat Kv1.1 α-subunit (50 mer) was purchased and purified by reverse-phase chromatography from Eurogentec S.A. The antisense sequence was 5’-TGACCTGGAGCGGCCGAAGCCTCGTCTGCATTCTC-3’ and was chosen to target the 5’-coding regions of the rat Kv1.1 α-subunit mRNA at residues 4–53 of the published cDNA sequence (Baumann et al. 1988). The sense oligodeoxynucleotide was used as a negative control. The specificity of the probes was evaluated with the public domain program:

**Table 2. Statistical significance**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Session 2–1 h</th>
<th>Session 2–4 h</th>
<th>Session 3–1 h</th>
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<td></td>
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<td>C/N</td>
<td>P5/N</td>
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<td>Dorsal hippocampus</td>
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<td>Dentate gyrus</td>
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<td>CA3 field</td>
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<td>CA1 field</td>
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<td>Temporal analysis</td>
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<td>Pseudoconditioned group</td>
<td>S2–1 h/ S2–4 h</td>
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<td>Conditioned group</td>
<td>S2–1 h/ S2–4 h</td>
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(C) conditioned group; (P) pseudoconditioned group; (N) naive group.
In situ hybridization and autoradiography

Because the quantification of the in situ hybridization signal requires analyzed brain section labeling to have been performed together, two in situ hybridization experiments were conducted. A first experiment collected brain sections of the three groups for one brain level and for one sacrifice time (i.e., 1 h after the second session, 4 h after the second session, and 1 h after the third session). A second experiment collected brain sections of the three sacrifice times for each brain level and for one group (i.e., naive control, preconditioned, and conditioned rats). Both experiments were conducted using exactly the same in situ hybridization protocol.

All solutions used for in situ hybridization were treated with diethylpyrocarbonate and autoclaved to avoid RNAse degradation. Slide-mounted sections were post-fixed for 5 min in 3% paraformaldehyde and were rinsed twice (each wash lasting 5 min) in a phosphate saline buffer (PBS). The sections were then incubated in pre-hybridization buffer containing 2 x standard saline citrate solutions (SSC) (twice, 5 min) and 1 x Denhardt’s solution (30 min), then rinsed in 2 x SSC (10 min). The sections were then acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine and treated for 30 min in 0.1 M Tris-glycine before being dehydrated in ethanol and air dried.

For DNA probes, each section was covered with labeled probe (5 x 10^6 cpm/sec) diluted in 35 μl of hybridization solution (4 x SSC containing 50% formamide, 10% dextran sulfate, 1 x Denhardt’s solution, 5 mg/ml Escherichia coli tRNA, and 2.5 mg/ml sheared salmon sperm DNA) and incubated for 12 h at 42°C in humid chambers. Sections were then rinsed in cold 2 x SSC, then treated successively with 1 x SSC (room temperature, 10 and 40 min), then in 1 x SSC (45°C, 10 and 40 min) and in 0.1 x SSC (42°C, 15 and 45 min). Sections were rinsed three times with 50% formamide in 2 x SSC (45°C, 5, 20, and 35 min) to decrease the background signal and in 1 x SSC (room temperature, 3 min). Sections were then dehydrated with a graded ethanol series and air dried.

Slides were exposed to Kodak Bio-Max MR-1 film for 11 d. Exposure times were adjusted to avoid film saturation. The X-ray films were developed with Kodak GBX solution for 3 min at 20°C, rinsed, and fixed. In the first experiment, same brain level sections from naive and experimental groups of animals for one learning stage were performed together in the same in situ hybridization and exposed side by side on the same autoradiographic film. In the second experiment, same brain level sections from different stages of learning for one experimental group were performed together in the same in situ hybridization and exposed side by side on the same autoradiographic film.

Autoradiograms data analysis

Film autoradiograms were scanned and the hybridization signal quantified using NIH image software by observers blind to the conditions of the experiment. The gray levels corresponding to mRNA-labeling levels were quantified using plastic standards (1°C, Amersham) to calibrate 218 relative concentration. mRNA-labeling levels are expressed in relative units. A mean level value was calculated for each brain structure of each hemisphere, from three unilateral measurements in each animal, and the mean ± S.E.M. was then calculated. The rat brain regions were identified and named using the rat brain atlas of Paxinos and Watson (1986). No specific hybridization was observed in negative control sections (sense probe); i.e., these sections produced a weak, but uniform labeling signal that did not follow neuroanatomical landmarks (Fig.2).

Statistical analysis

Statistical analysis was performed with the SPSS/PC statistical software by SPSS Inc. Differences between groups were assessed using an ANOVA followed by a Scheffé post hoc test to compare within multiple groups. Statistical significance for each analysis is indicated in the relevant results section. Simple-regression analysis was used to identify correlations between individual learning to changes in mRNA level. Significance was set at P < 0.05.

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References


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Kv1.1 subunit mRNA down-regulation during learning


