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BRAIN RESEARCH

Research Report

$GABA_A$ receptors in deep cerebellar nuclei play important roles in mouse eyeblink conditioning

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ARTICLE INFO

Article history:
Accepted 15 June 2008
Available online 28 June 2008

Keywords:
Eyeblink conditioning
Cerebellar nuclei
GABA
Mouse

ABSTRACT

The neural circuitry of eyeblink conditioning in rabbits has been studied in detail, however, the basic knowledge of eyeblink conditioning in mice remains limited. In the present study, we examined the role of the deep cerebellar nuclei (DCN) in mice in delay eyeblink conditioning and rotor rod test performance by using the γ-aminobutyric $acid_A$ ($GABA_A$) receptor agonist muscimol (MSC) and the $GABA_A$ receptor antagonist picrotoxin (PTX). Bilateral injections of MSC and PTX into the DCN significantly impaired motor coordination in the rotor rod test, however the performance recovered within 24 h after the injections. Bilateral injection of MSC and PTX significantly impaired learned eyeblink responses (LER) during the acquisition test. MSC-injected mice could not acquire LER, however, PTX-injected mice acquired LER latently, suggesting the distinctive effect of these drugs in DCN. Bilateral injection of MSC and PTX also impaired the retention of acquired LER during a 7-day performance test. Furthermore, ipsilateral injections of MSC and PTX impaired the acquired LER as much as bilateral injection of them. Contralateral MSC injections also impaired the expression of LER, but contralateral PTX injections only partially impaired eyeblink conditioning. These results suggest that $GABA_A$ receptors in bilateral DCN play important roles in both the acquisition and the expression of mouse eyeblink conditioning, and that $GABA_A$ receptors not only in ipsilateral but also in contralateral DCN are critical for the expression of LER.

1. Introduction

Eyeblink conditioning is one type of associative learning and is a useful paradigm to analyze the neural substrates underlying learning and memory (for review, see Christian and Thompson, 2003). The eyeblink conditioning paradigm involves the paired presentation of a neutral conditioned stimulus (CS), such as a tone, and an unconditioned stimulus (US), such as a corneal air puff or a periorbital shock. In delay conditioning (a typical eyeblink conditioning paradigm) the CS and US overlap temporally, although CS onset precedes US onset. Many studies using rabbits, rats, mice, and humans indicate that the cerebellum is essential for the acquisition and retention of conditioned responses (CR) in delay eyeblink conditioning (Kim and Thompson

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Abbreviations: aCSF, artificial cerebrospinal fluid; CR, conditioned responses; CS, conditioned stimulus; DCN, deep cerebellar nuclei; EMG, electromyogram; GABA, γ-aminobutyric acid; LER, learned eyeblink responses; LTD, long-term depression; MSC, muscimol; pcd, Purkinje cell degeneration; PKC, protein kinase C; PTX, picrotoxin; RNB, reversible transmission blocked; SD, standard deviation; SEM, standard error of the mean; SER, spontaneous eyeblink responses; STR, startle eyeblink responses; US, unconditioned stimulus

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doi:10.1016/j.brainres.2008.06.079

1997; Green and Woodruff-Pak 2000; Attwell et al., 2002; Christian and Thompson, 2003, Freeman et al., 2005; Gerwig et al., 2005).

The neural circuitry for eyeblink conditioning has been established mainly from cumulative research on rabbits (Christian and Thompson, 2003). Cerebellar mossy fibers and climbing fibers convey the CS and US, respectively, both being transmitted to deep cerebellar nuclei (DCN) and cerebellar cortex (Svensson te al., 1997; Mauk et al., 1986; Medina et al., 2002, review). Non-reversible lesions or reversible inactivation of the DCN impairs the acquisition and expression of the CR in rabbit eyeblink conditioning (Steinmetz et al., 1992; Krupa et al., 1993; Krupa and Thompson, 1997). Non-reversible lesions or reversible inactivation of the cerebellar cortex also impairs the eyeblink conditioning performance (Yeo et al., 1985; Attwell et al., 2001). Studies also suggest that the cerebellar cortex is involved in the timing of eyeblink responses (McCormick and Thompson, 1984; Garcia and Mauk, 1998; Perrett, 1998). These results obtained in rabbits reveal that both the DCN and cerebellar cortex are critically involved in eyeblink conditioning.

DCN have also reported to play an important role in eyeblink conditioning in rats (Kleim et al., 2002; Nolan et al., 2002; Freeman et al., 2005). In mice, however, the neural circuitry underlying eyeblink conditioning is yet to be elucidated. Much work has been done to examine the neural network and/or molecular mechanisms for mouse eyeblink conditioning using cerebellum-specific genetically modified mice. For example, Purkinje cell degenerative (pcd) mice (Chen et al., 1996), GluRδ2-deficient mutant mice (Kishimoto et al., 2001b), Purkinje cell-specific protein kinase C (PKC) inhibitor–overexpressing mice (Koekkoek et al., 2003), and granule-cell specific reversible neurotransmission blocked (RNB) mice (Wada et al., 2007) cannot acquire the CR in delay eyeblink conditioning. Furthermore, GluRδ2-(Kashiwabuchi et al., 1995) and PKC inhibitor-overexpressing mice (De Zeeuw et al., 1998) show impaired cerebellar long-term depression (LTD), a synaptic substrate candidate for eyeblink conditioning (for review, see Ito, 2002).

These behavioral and electrophysiological deficits in genetically modified mice indicate that the cerebellar cortex is critically involved in the acquisition and/or expression of eyeblink conditioning (Kashiwabuchi et al., 1995; De Zeeuw et al., 1998; Kishimoto et al., 2001b; Koekkoek et al., 2003). However, the role of the DCN in eyeblink conditioning in mice remains uncertain. DCN lesion studies using pcd mice (Chen et al., 1999) and RNB mice (Wada et al., 2007) showed a complete loss of the acquisition of CR. On the other hand, both wild-type mice and PKC inhibitor-overexpressing mice, in which the DCN were lesioned bilaterally after eyeblink training, displayed a remnant CR having distorted timing and magnitude (Koekkoek et al., 2003). Thus, these apparent conflicting results warrant further investigation into the role of the DCN in eyeblink conditioning in mice.

The main pathway from Purkinje cells to the DCN is GABAergic (Ito, 2002). However, the role of GABA receptors in the mouse DCN has not been elucidated. Thus, in the present study, we investigated the role of $GABA_A$ receptors in the DCN during delay eyeblink conditioning in C57BL/6 mice. First, we examined how injections of the $GABA_A$ agonist muscimol (MSC) and the $GABA_A$ antagonist picrotoxin (PTX) affected motor coordination. Then, we examined the acquisition and retention of eyeblink conditioning in the presence and absence of MSC or PTX. Finally, to examine the role of the individual cerebellar hemispheres, we investigated the effects of unilateral (ipsilateral and contralateral) injections of MCS and PTX on eyeblink conditioning.

2. Results

2.1. *Identification of cannula tip location in the DCN*

To determine how $GABA_A$ receptors in the DCN (especially in the interpositus nucleus) affect motor coordination and eyeblink conditioning, we injected (by cannulae) the $GABA_A$ agonist MSC or the $GABA_A$ antagonist PTX into the DCN. An aqueous solution of neutral red (0.2 μl × 2) was injected into the DCN bilaterally after the final drug injection to identify the location of the cannulae tips during histological verification. The diffusion of the dye was also used to estimate the approximate spatial extent of the drug diffusion. The location of the drug injections were verified before analyzing the behavioral data (Fig. 1): Mice were excluded from behavioral analyses if the cannulae tips were outside of the DCN or if the dye had diffused into the brain stem. Fig. 1A shows a representative brain section from a case deemed valid for behavioral analysis. The location of the cannulae tips in MCS-, PTX-, and aCSF-injected mice are shown in Fig. 1B, indicating that consistent DCN injections, including those in the interpositus nucleus, were obtained in all three groups of mice.

2.2. *Effects of MSC and PTX injections on motor performance*

The functional impairment of the DCN caused by MSC and PTX was determined by examining motor coordination and balance in injected mice. Immediately after injection, MSC and PTX induced apparent ataxia. To quantitatively analyze the extent of the motor deficits caused by MSC and PTX injections, we subjected the mice to a rotor rod test, in which the mouse was placed on a constantly rotating rod (8 rpm). Fig. 2 shows the average latency to fall in this test. All groups of mice learned the rotor rod task in five trials. MSC or PTX injections given on days 1, 5, and 8 impaired performance: day 1 (control, 119.9 ± 0.05; MSC, 0.47 ± 0.38; PTX, 5.3 ± 1.7 s); day 5 (control, 119.6 ± 0.43 MSC, 0.29 ± 0.23; PTX, 2.4 ± 1.1 s); day 8 (control: 119.6 ± 0.42; MSC, 0.0 ± 0.0; PTX, 3.8 ± 1.7 s). The mean latencies of MSC- and PTX-injected mice were significantly shorter than those observed in control mice (aCSF-injected mice and uninjected mice), as assessed by Mann–Whitney *U* test analyses (control vs. MSC, all sessions, $Ps < 0.001$; control vs. PTX, all sessions, $Ps < 0.01$).

Recovery from the drug injections was examined 24 h after the injections. All groups performed similarly to their levels during the last three trials of training on day 1 (Fig. 2; days 2, 6, and 9). This was verified with the Kruskal–Wallis test, which showed no fall latency differences among the three groups (all tests, $Ps > 0.05$). These results indicate that injections of MSC and PTX into the DCN transiently impaired the DCN function, manifested as disturbed motor coordination. These impairments were not permanent, as recovery occurred within 24 h after drug injection.