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Research report

Knockout of c-Jun N-terminal kinases 1, 2 or 3 isoforms induces behavioural changes

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HIGHLIGHTS

- Behaviour was compared between JNK1, JNK2 and JNK3 knockout (ko) mice and appropriate wild type control.
- JNK1 ko showed a more pronounced explorative behaviour.
- JNK2 ko and JNK3 ko differed from JNK1.
- JNKs are involved anxiety, exploration and learning consolidation.

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ABSTRACT

c-Jun N-terminal kinases (JNKs) are central and ubiquitous mediators of cellular signaling for both physiogical-regenerative and pathological-apoptotic processes. Their impact on degeneration or inflammation is well documented, but so far little is known about their roles in higher brain functions. The more, the contribution of individual JNK isoforms remains obscure so far.

Here we have tested the behaviour of JNK1, JNK2 and JNK3 knockout (ko) mice in elevated plus maze (EPM), open field (OF), novel object recognition memory (NORM) test and Morris water maze (MWM). Compared with wild type C57BL/6N mice JNK ko mice revealed significant differences. Taken together the data on anxiety, exploration and learning indicate that JNK1 ko mice displayed a stronger explorative behaviour and that knockout of JNK2 or JNK3 showed a tendency of behaviour opposite to that of JNK1 ko mice. This pattern reminds of the impact of individual JNK ko on neurodegeneration. This is the first comparative study on the impact of individual JNK ko on behavioural parameters.

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1. Introduction

The c-Jun N-terminal kinases (JNKs) are ubiquitous and multifunctional enzymes in cellular function. This is also true for the nervous system where JNKs are important regulators of physiological processes such as brain development, repair or synaptic plasticity at the one hand, but also of apoptosis and degeneration at the other hand [1–6]. Described initially as stress kinases [7–9], JNKs have been studied mostly in the context of cellular stress and apoptotic cell death, e.g. following heat shock, formation of radicals, DNA damage [8,9], ischemia or as trigger of neurological diseases such as Alzheimer's disease, Parkinson's disease, excitotoxicity, stroke or neuroinflammation (reviewed by [3,6]).

Much less, however, is known about the role and mode of action of JNKs under physiological conditions. There is raising evidence that JNKs are involved in synaptic plasticity [10,11], regeneration or embryonic development of the nervous system [12,13]. Thus, inhibition of JNK blocks outgrowth of neurites and axons [14,15]. Importantly, human neurological disorders such as dysautonomia [16] or severe disturbances of brain development have been linked with loss of function of JNK [17].

All three JNK isoforms JNK1, JNK2 and JNK3 are expressed in the brain. Only few data are available about the individual actions of specific JNK isoforms in brain functions, mainly in neurodegenerative pathology. Very few is known about the specific involvement in cognitive and behavioural functions. The pronounced basal expression, the regulation of gross and fine brain architecture and the

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central control of gene expression and metabolic homeostasis strongly suggest a relevant role of JNKs in higher brain functions and behaviour. Actually, JNKs are involved in the hippocampal long-term potentiation (LTP) in adult mice [18], but not in young mice [19,20], and deletion of JNK3 enhanced the excitation threshold of kainic acid [21]. On the other hand, JNKs are described as inhibitors of LTP by beta-amyloid (Abeta) [19] or by lipopolysaccharide-induced cytokines [22]. Beyond LTP, JNKs are modulators of long-term depression (LTD). The JNK inhibitor SP600125 significantly attenuated N-methyl D-aspartate (NMDA)–induced LTD [23] and metabotropic glutamate receptors (mGluR)-mediated LTD was impaired in JNK1-deficient mice [20]. However, unspecific inhibition of all JNK-isoforms by SP600125 ignores the probably differential and opposite actions of individual JNK isoforms as we know from various studies on neurobiological functions [6,24–26].

Summarizing, JNK inhibition might be critical to physiological processes underlying cognitive functions.

Here we tested in a variety of behavioural patterns and reaction tasks with emphasis on tests of learning and memory, in JNK1, 2 and 3 ko mice. Our data indicate for the first time the isoform-dependent role of JNKs in explorative behaviour, but not in memory formation.

2. Materials and methods

2.1. Animals

All experimental procedures were conducted in accordance with the German Law of Animal Protection. The experiments were carried out on each 9 male C57BL/6N mice of the knockout variants JNK1, JNK2, JNK3 and age matched male wild-type mice (CTRL). Animals were 3 months old. Genetic inactivation of JNK1, JNK2 or JNK3 in mice has been described in detail elsewhere [13,27,28]. Mice were backcrossed to the C57BL/6N strain for at least 10 generations and can therefore be considered of almost uniform genetic background. All mice were housed in plastic cages in a sound and light attenuated housing room under a constant light-dark cycle (light phase 08:30–20:30; 21° ± 1 °C) with ad libitum access to food and water. The behaviour was video recorded on the hard disk of a personal computer using the Noldus software EthoVision XT (www.noldus.com). Behaviour was analyzed off-line automatically or by an observer blinded for the genotype. After the last day of the experimental procedure all animals were weighed.

2.2. Western blot analysis

Western blot analysis was performed as described in detail recently [26]. Mice were decapitated, the brains were rapidly removed and snap-frozen in liquid nitrogen. Thereafter, the tissue was homogenized in 0.2– −0.4 ml of lysis buffer containing 50 mM Tris–HCl (pH 7.5), 8 mM MgCl2, 0.5 mM EDTA, 5 mM EGTA, 250 mM NaCl, 0.01 mg/ml Pepstatin, 0.01 mg/ml Leupeptin, 0.01 mg/ml aprotinin and 1 mM phenylmethylsulphonyl fluoride (Sigma Chemical, USA). The lysates were frozen and thawed three times and incubated on ice for 30 min. After sonication for 10 s, the tissue samples were centrifuged at 15,000 × *g* for 15 min at 4 °C. Protein concentrations were determined using Dye Reagent, a variant of Bradford's colorimetric assay (Roth, Germany).

Total tissue extracts were separated on a 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), and transferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 4% non-fat dry milk and incubated with the primary antibodies overnight at 4 °C. After three washing steps with TBST, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody for 30 min. All western blots were developed using the chemiluminescence system and Hyperfilm ECL (GE Healthcare). Blots were stripped in 2% SDS, 62.5 mM Tris nad 100 mM 2-mercaptoethanol for 30 min at 50 °C, washed with TBST and blocked again. To normalize for the protein content of each line and to confirm equal loading, all membranes were finally stained with Ponceau S. Primary antibodies against following targets were purchased from the indicated sources: mouse anti-JNK1 monoclonal antibody (BD Pharmingen; San Diego, CA), rabbit anti-JNK2 monoclonal antibody (Epitomics; Burlingame, CA), rabbit anti-JNK3/SAPK1b monoclonal antibody (Upstate; Lake Placid, NY). All Western blottings were performed with samples from two dependent experiments.

2.3. Behaviour

2.3.1. Elevated plus maze

Anxiety behaviour was assessed in the elevated plus maze (EPM). The EPM was made of dark grey plastic and was mounted 90 cm above the dark grey floor of a sound and light attenuated recording chamber. The center (5 cm × 5 cm) of the maze was connected to four arms (42.5 cm × 5 cm); the walls of the two closed arms were made of white opal plastic (39 cm high). The mice were put on the maze for 5 min. To investigate anxiety, exploratory and locomotor activity, following parameters were measured: the total time spent in the center, the open and closed arms, the total distance moved and mean velocity, immobility time and the frequency of entering center.

2.3.2. Open field

Activity monitoring was conducted for 5 min in a square shaped (50 cm × 50 cm) open field (OF) made of dark grey plastic. To investigate anxiety, exploratory and locomotor behaviour, following parameters were assessed: total distance moved, mean velocity, immobility time, the time spent in border zone (borders and corners), corners, and centre zone.

2.3.3. Novel object recognition memory

A well established test to investigate non-spatial, and thus hippocampus-independent, memory performance is the novel object recognition memory (NORM) test [29]. This test utilizes the natural tendency of rodents to explore novel objects more than familiar ones. In our study an experiment comprised two sessions of each 300 s performed in the animals' home cage: During the first session two identical objects were presented to the animal and one hour later (test session) one of the familiar objects was replaced by a novel one. The following parameters were assessed: For both sessions the cage exploration as percentage of the total time [%] and a recognition index was calculated for each animal and expressed as a ratio: (time novel object) × 100/[(time novel object) + (time familiar object)]. The recognition index is around 50% when animals did not remember the familiar object, while recognition indices greater than 50% support that animals remembered the familiar object and spent more time with the novel object [30].

2.3.4. Morris water maze

The Morris Water Maze (MWM) is a classical test to investigate spatial and thus hippocampus-dependent memory consolidation [31]. The test was performed in a dark grey circular pool (diameter 120 cm) filled with water at a temperature of 24° ± 0.5 °C. Four landmarks (black geometrical symbols) were placed around the water maze. The maze was divided into four quadrants. In the target quadrant a transparent escape platform (diameter 10 cm) was placed 1 cm beneath the water surface. Two independent MWM experiments with different target locations were performed (with an interval of two weeks) for all animals. Each experiment consisted of three consequent training days with three training sessions each and one test (reversal) day with two sessions. When animals did not find the platform during the training sessions within 90 s they were placed on it for additional 10 s. On the test (reversal) day the platform was removed and two test trials were run with duration of 60 s with starting points from two randomly chosen quadrants. The following parameters were determined for each session: the escape latency until the mice reached the platform or its position (in tests), the mean distance to the platform, the time spent in the border zone as percentage of the recording time (in %), the frequency of platform crossings in the test sessions and the total distance moved.

2.4. Statistical analysis

All statistical analyses were performed with the standard software package GraphPad Prism 4 (www.graphpad.com). The number of animals per group was $n = 9$. Either one-way ANOVAs were run to detect genotype differences, or 2-factor-repeated-measurements (RM) ANOVAs with the factors genotype and time or experimental were run when appropriate post hoc Bonferroni *t*-tests were calculated. Level of significance was set at $p < 0.05$. For all behavioural parameters not mentioned in the result part statistical analyses revealed no significant effects ($p \geq 0.05$).

3. Results

3.1. Bodyweight

Genetically induced knockout of the different JNK variants affected the body weight (Table 1). One-way ANOVA gave a significant effect of JNK ko ($F_{(3,32)} = 14.17$; $p < 0.0001$; Bonferroni post hoc test: CTRL vs. JNK2 ko $p < 0.05$; CTRL vs. JNK3 ko $p < 0.05$; JNK1 ko vs. JNK3 ko $p < 0.0001$; JNK2 ko vs. JNK3 ko $p < 0.0001$).The JNK3 ko had a significant higher body weight compared to the other groups.

3.2. Western blot analysis

To confirm single JNK ko genotypes we first determined the basal expression of JNK isoforms in the hippocampus of the brain of JNK deficient mice by Western blotting (Fig. 1A). The deficiency of an individual JNK isoform resulted in the complete absence

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