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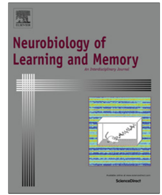

Short communication

Short-term treatment with flumazenil restores long-term object memory in a mouse model of Down syndrome

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ABSTRACT

Down syndrome (DS) is a common genetic cause of intellectual disability yet no pro-cognitive drug therapies are approved for human use. Mechanistic studies in a mouse model of DS (Ts65Dn mice) demonstrate that impaired cognitive function is due to excessive neuronal inhibitory tone. These deficits are normalized by chronic, short-term low doses of $GABA_A$ receptor ($GABA_AR$) antagonists in adult animals, but none of the compounds investigated are approved for human use. We explored the therapeutic potential of flumazenil (FLUM), a $GABA_AR$ antagonist working at the benzodiazepine binding site that has FDA approval. Long-term memory was assessed by the Novel Object Recognition (NOR) testing in Ts65Dn mice after acute or short-term chronic treatment with FLUM. Short-term, low, chronic dose regimens of FLUM elicit long-lasting (>1 week) normalization of cognitive function in both young and aged mice. FLUM at low dosages produces long lasting cognitive improvements and has the potential of fulfilling an unmet therapeutic need in DS.

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1. Introduction

Down's syndrome (DS) is the most common genetic form of intellectual disability (ID) with a prevalence of approximately 1/750 live births (Morris & Alberman, 2009). DS is caused by triplication of all or part of chromosome 21 resulting in differential developmental and physiological effects (Nadel, 2003). In recent decades, general health, quality of life, and expected lifespan of individuals with DS have improved significantly (Yang, Rasmussen, & Friedman, 2002). Moreover, there have been several efforts (whether behavioral or pharmacological interventions) to alleviate the ID in people with DS (de la Torre et al., 2016). Transgenic mice engineered to possess extra copies of mouse genes syntenic to Hsa21 have made it possible to investigate molecular, cellular and functional consequences of triplication of genes found on Hsa21 (Gardiner et al., 2010). Ts65Dn mice, are trisomic for a segment of mouse chromosome 16 containing >150 genes that are syntenic to genes on Hsa21 but also 60 genes not related to Hsa21 (Creau, 2012; Davisson, Schmidt, & Akeson, 1990). Young Ts65Dn mice display key features of DS including cognitive deficits (Gupta, Dhanasekaran, & Gardiner, 2016). Older Ts65Dn mice develop neural alterations indicative of Alzheimer's disease (AD), including abnormal APP (beta-amyloid precursor protein) processing and neuronal losses in basal forebrain and locus coeruleus (Salehi et al., 2006, 2009). Ts65Dn mice are a well accepted and widely used model of DS even though more complete genetic models of DS are becoming available (Belichenko et al., 2015).

Work by Fernandez et al. (2007) led to the hypothesis that learning disability associated with DS was caused by tonic overinhibition affecting neural plasticity processes in certain brain circuits such as the hippocampus. This hypothesis was supported by prior electrophysiological studies showing impaired hippocampal long-term potentiation (LTP) in Ts65Dn mice. A possible explanation of those findings was increased inhibition (Costa & Grybko, 2005; Kleschevnikov et al., 2004; Siarey et al., 1999, 2006). Subsequently, Fernandez et al. (2007) showed that chronic treatment of Ts65Dn mice with several $GABA_AR$ antagonists resulted in long-lasting improvements in cognitive functioning and also in hippocampal LTP (Fernandez et al., 2007). Continued work on Ts65Dn mice extensively characterized the procognitive therapeutic potential of the $GABA_AR$ antagonists, pentylenetetrazole (PTZ), showing that its procognitive effects are seen at very low dosages (0.3 mg/kg). Acute administrations just prior to training had a strong but short-lived procognitive effect, but a chronic regimen of daily

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doses for two weeks produced improvements in learning ability that lasted weeks to months (Colas et al., 2013; Rueda, Florez, & Martinez-Cue, 2008). These procognitive effects were seen in young, adult, and old Ts65Dn mice ruling out a developmental effect. However, therapy was only effective when delivered at a certain circadian phase corresponding to the main sleep phase (Colas et al., 2013). Supportive evidence for procognitive effects of low (non-epileptic) doses of $GABA_AR$ antagonists have been extended to AD model mice (Yoshiike et al., 2008).

Cumulative results showing that short-term chronic treatments with $GABA_AR$ antagonists produce long-lasting procognitive effects in DS and AD model mice suggest that such a drug could benefit humans afflicted with DS. However, no compound used in these studies to date have FDA approval. PTZ is a strong candidate for clinical trials as it had a long history of safe use in humans (Aschenbach, 1956; Deitrick, 1967; Gross & Finn, 1954; Kapernick, 1957). However, it was removed from the approved list of drugs because of lack of evidence for efficacy in senile dementia, which was its primary indication. Drugs blocking the alpha-5 subunits of the benzodiazepine receptors have also been shown to improve memory but effects were not long lasting (Braudeau et al., 2011). Flumazenil (FLUM) is a $GABA_AR$ antagonist that is in current clinical use (Bentué-Ferrer, Bureau, Patat, & Allain, 1996). FLUM is of interest because unlike PTZ which is a non-competitive $GABA_AR$ antagonist, FLUM is a competitive antagonist acting at the benzodiazepine binding site. It has the properties of rapid brain availability of minutes and a short half life of less than 1 h (Bentué-Ferrer et al., 1996). Could a chronic, but short-term, treatment with such a short acting drug have the same long-lasting effects as has been documented for PTZ?

2. Methods

2.1. Animals and genotyping

Segmental trisomic 16 (Ts65Dn) mice were obtained by mating female carriers of the 17^{16} chromosome (B6EiC3H – a/ATs65Dn) with C57BL/6J Ei × C3H/HeSnJ (C3H) F1 hybrid males (Reeves et al., 1995) and produced either at Jackson West Laboratories, (Davis, CA) or in our colony. Mice used in our studies were Ts65Dn males (TS) on the B6/C3H background with diploid (2N) littermates as controls. Mice were maintained at 23 ± 2 °C on a 12:12 Light-Dark schedule and had access to food and water *ad libitum.* All experimental procedures were approved by the Stanford University IACUC and were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used and to minimize their discomfort as our aim was to study the behavior of non-stressed animals. The 2N and Ts65Dn mice were genotyped using real-time quantitative PCR with *App*- and *Apob*-specific TaqMan probes (Applied Biosystems). Mice carrying the retinal degeneration (*Rd*) allele were excluded from experiments (Blank et al., 2011). Mice were randomly assigned to experimental groups.

2.2. Drug administration and scheduling

Flumazenil (Ro 15-1788) was purchased from Sigma Chemical Co. (St. Louis, MO). FLUM stock solutions were made by dissolving 6.2 mg FLUM in 3.1 ml normal saline (0.9%) containing 10% DMSO. With an injection volume of 0.1 ml, the resulting FLUM dose for a 40 g mouse was 5 mg/kg. The volume was adjusted to deliver this same dose to mice of different sizes. Normal saline containing 10% DMSO was the vehicle (VEH). Chronic regimen: Drug injections were delivered intraperitoneally (IP) to all subjects within 1 h at the middle of the light phase (6 h after light onset). The chronic treatment protocol was daily IP doses of FLUM (5 mg/kg) or VEH for two weeks followed by 8 days without treatment. After the no-treatment phase, mice were assessed for learning and memory using the novel object recognition task (NOR) as described below. The acute treatment protocol involved IP injections of FLUM 10 mg/kg or VEH 10-20 min prior the NOR training. Timing and dosage were based on pilot studies and previous work (Lal, Kumar, & Forster, 1988). Independent groups of mice were used in experiments done on different aged mice and in experiments using chronic or acute protocols.

2.3. Behavioral testing

The behavioral test used in this study for assessing long-term memory was novel object recognition or NOR (Dere, Huston, & De Souza Silva, 2007), carried out in arenas (50 × 50 × 50 cm) resting on an infra-red emitting base. Behavior was recorded by an infrared-sensitive camera placed 2.5 m above the arena. Behavioral testing was carried out in dim light within 2 h in the middle of the light phase (6 h after light onset). Data were stored and analyzed using Videotrack software from ViewPoint Life Sciences, Inc. (Montreal, Canada). On the day before NOR training, mice were habituated to the open arenas. NOR is based on propensity of mice to explore a novel object versus a previously experienced object when allowed to explore freely. Procedures used in Colas et al. (2013) were used: for NOR training, two identical objects were placed in the arena and animals were allowed to explore them for 10 min. Testing occurred 24 h later in the same arena, but one of the original objects used during training was replaced by a novel object. Objects were of similar dimensions and prior testing showed that they did not elicit spontaneous preferences. Testing sessions were 7 min after which the objects and arenas were cleaned with 10% ethanol. Object exploration was measured by time spent with the nose directed at and within 2.5 cm from the object. A discrimination index (DI) for each animal in each trial was calculated as ratio of time spent exploring the novel object over total time spent exploring both objects × 100%. The DIs were averaged among the groups of mice by genotype/treatment/condition. The DIs should be non-significantly different from 50% in the training session, and significantly increased in test sessions vs training sessions if novelty is detected (Ruby et al., 2008).

2.3.1. Experimental groups and conditions

Three independent groups of mice were studied.

2.3.1.1. Experiment-1 – Chronic treatment in young mice. Four groups of 10 mice, 2–3 months of age were used. Treatment groups were: 2N VEH, 2N FLUM, TS VEH, TS FLUM. All mice were treated in parallel with daily IP injections of VEH or FLUM (5 mg/kg) at mid-light phase for 2 weeks. One week following treatment the first 24 h NOR testing was performed, and 24 h NOR testing was repeated 4 weeks later.

2.3.1.2. Experiment-2 – Chronic treatment in older mice. Two groups of 10 2N or TS mice, 8–10 months old were used. These groups first received daily IP VEH injections at mid-light phase for two weeks and were then subjected to 24 h NOR testing one week following the last injection. Subsequently, the two groups were then treated with daily, mid-light phase IP injections of FLUM (5 mg/kg) for two weeks. A week after last injection, mice were subjected to 24 h NOR testing with different sets of objects.

2.3.1.3. Experiment-3 – Acute treatment with cross-over design. Two groups of 2N (n = 20) and TS mice (n = 15), 4–8 months old were pretreated IP with VEH or FLUM 10–20 min before the training ses-

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