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NMDA receptor involvement in the effects of low dose domoic acid in neonatal rats

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Summary. We have previously reported that neonatal rats display enhanced sensitivity to domoic acid relative to adults, and that perinatal injections of low doses of domoic acid alter early associational learning in the newborn rat. The current study was designed to further investigate the effects of low dose domoic acid on neonatal odour conditioning and to determine if the observed effects are due in part to an action on NMDA receptors. Groups of rat pups were conditioned to a novel odour on postnatal day (PND) 8, injected with 20 μg/kg domoic acid either alone, or in combination with the NMDA antagonist CPP (or appropriate controls), daily from day 8–14, reexposed to the conditioning odour or a novel odour on day 9, and tested for odour preference on day 13 using a standard 3-choice paradigm. Results indicated that rats treated with domoic acid spent significantly more time over the conditioning odour than did saline-treated rats when tested on PND 13. This effect was antagonized by concomitant injection of CPP, indicating an involvement of NMDA receptors in the actions of DOM in this paradigm. Rats injected with either saline or CPP alone showed the opposite effect, i.e. a preference for the alternate odour. The results indicate that a very low dose of DOM produces a conditioned odour preference in neonatal rats and that this effect is due in part to NMDA receptor involvement, thereby emphasizing a role for both kainate and NMDA glutamate receptors in implicit memory.

Keywords: Domoic acid – Kainate receptors – NMDA receptors – Odour conditioning – Postnatal development

Introduction

Glutamate is the primary excitatory amino acid in the mammalian central nervous system and is known to be integrally involved in many forms of neural plasticity including learning and memory (Ozawa et al., 1998). Normal glutamatergic function is also critical to brain development, and accordingly the synthesis and degradation of glutamate, as well as the expression of glutamate receptors, is tightly regulated during CNS development (McDonald and Johnston, 1990; Paschen et al., 1997). Glutamate receptors are classified as either metabotropic or ionotropic, with ionotropic receptors being further subdivided into N-methyl-d-aspartate (NMDA), AMPA (2-amino-3-[3-hydroxy-5-methylisoxazol-4-yl]propionic acid) and kainate subtypes (for reviews see Jorgensen et al., 1995; Bleakman and Lodge, 1998). The sensitivity of the developing CNS to glutamatergic compounds has been relatively well characterized with respect to NMDA receptors. Using behavioural measures as sensitive indicators of receptor activation, many studies have reported altered learning and memory in neonatal rats following administration of NMDA receptor agonists and/or antagonists (Lincoln et al., 1988; Weldon et al., 1997; Mickley et al., 2000). The contribution of AMPA and kainate receptors to similar measures of developmental plasticity *in vivo*, however, has been comparatively less well studied. Domoic acid (DOM) and kainic acid (KA) are generally considered to be agonists for kainate receptors both *in vitro* (Johansen et al., 1993; Fernandez-Sanchez and Novelli, 1996) and *in vivo* (Tasker et al., 1996; Doucette et al., 2000) with DOM displaying higher affinity for receptor configurations that include the GluR5 and GluR6 proteins (Verdoorn et al., 1994; Tasker et al., 1996) whereas KA binds with higher affinity to the KA1 and KA2 subunit proteins (Werner et al., 1991; Herb et al., 1992). These pharmacological designations are not, however, absolute in terms of either receptor specificity or biological response, because kainate receptor activation is known to modulate NMDA receptor function under certain conditions and a number of authors have reported that both DOM and KA can act pre-synaptically to facititate glutamate release causing NMDA receptor activation both

in vitro and *in vivo* (Novelli et al., 1992; Fernandez-Sanchez and Novelli, 1996; Berman and Murray, 1997; Tasker and Strain, 1998; Tasker et al., 2002).

We and others have previously reported that the newborn rat is exquisitely sensitive to the neurotoxic properties of DOM and KA (Xi et al., 1997; Doucette et al., 2000). Recently, we reported that administration of extremely low doses of DOM to rats during the second postnatal week of life resulted in no observable toxicity but did alter neonatal learning at 13 days of age as measured in a simple forced-choice odour conditioning paradigm (Doucette et al., 2003). Our objective in the current study was to expand upon these previous findings by investigating (a) whether the effect of DOM on day 13 reflects a positive association with the conditioning odour or an aversion to a novel odour, and (b) whether the effect was mediated in part by NMDA receptor activation.

Materials and methods

Experimental animals and injection paradigm

All studies were conducted using the offspring of untimed pregnant Sprague-Dawley rats (Charles River, Lasalle, PQ, Canada) with the day of parturition designated as postnatal (PND) 0. Within 24 hours of birth, litters were culled to 10 pups (5 male, 5 female where possible) and tail marked for identification. All drug conditions were represented within each litter. Dams and offspring were housed in standard polypropylene caging with wood chip bedding and the colony room was maintained at 22°C on a 12 hour light/dark (0700–1900 h) cycle with food and water available *ad libitum*. Pups were injected (s.c. in a volume of 10 ml/kg) daily from PND 8–14 with either saline, domoic acid (DOM) (20 μg/kg), the competitive NMDA receptor antagonist CPP (150 μg/kg), or combinations of DOM and CPP. Animals were observed continually for 1 hour post-injection and subsequently checked every 8 hours for overt signs of toxicity.

Drugs and reagents

Domoic acid was obtained from BioVectra dcl (Charlottetown, PE, Canada) and CPP [(±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid] was obtained from Sigma-Aldrich (St. Louis, MO, USA). Both drugs were dissolved in sterile (0.85%) saline.

Olfactory conditioning protocols

On PND 8, rat pups were injected and placed in a plexiglas conditioning chamber (29.5 × 18.6 × 12.5 cm) maintained at 37°C (nest temperature) and with a plastic mesh floor over top of 500 ml of bedding previously saturated with 3.0 ml of peppermint extract (Club House brand). Pups were left in the conditioning chamber for 30 minutes and monitored continuously for signs of distress (never noted). Following conditioning, pups were returned to their home cage and left undisturbed.

On PND 9 and 13, pups were tested for odour preference using a classical conditioning place preference test in a three choice configuration. The test chamber was divided into 3 inter-connected sections designated "odour", "neutral" and "no odour" (40/20/40 by area respectively). "Odour" sections contained 300 ml of bedding saturated with 1.8 ml of either the conditioning odour (peppermint) or an alternate odour (almond). "No odour" sections contained untreated bedding. Odour and No odour sections were divided by a "neutral" zone that had no bedding beneath the floor. Total time spent over each section was scored from videotape recordings with the experimenter blind to treatment. Each test session lasted 10 minutes.

Adherence to guidelines

All procedures were approved in advance by the UPEI Institutional Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Results

Overt signs of toxicity were not noted in any animal in any treatment group. After drug injection had been paired with peppermint odour on PND 8, each drug group was divided such that half the rats were tested using the familiar odour (peppermint) and half were tested using an alternate odour (almond). Examination of the time spent over peppermint vs almond on PND 9 using 2 way ANOVA indicated no significant drug × odour effect ($F\ [1,\ 59] = 2.44$, $p > 0.05$). These data indicate that within each drug group, pups displayed no innate preference for either odour, confirming our previous observations (unpublished) that there are no intrinsic differences between these extracts. PND 9 testing in this manner also ensured that all animals were handled equally while removing novelty (to either almond or the test chamber) as a confounding variable on PND 13. The results of odour testing on PND 9 did, however, reveal a significant drug effect as shown in Fig. 1. Rats treated with either saline or DOM alone spent considerably more time over odour (peppermint and almond combined) than did rats treated with CPP, either alone or in combination with DOM ($F\ [3, 59] = 5.34$, $p < 0.01$) (Fig. 1). Post-hoc analysis revealed that both CPP groups were significantly differ-

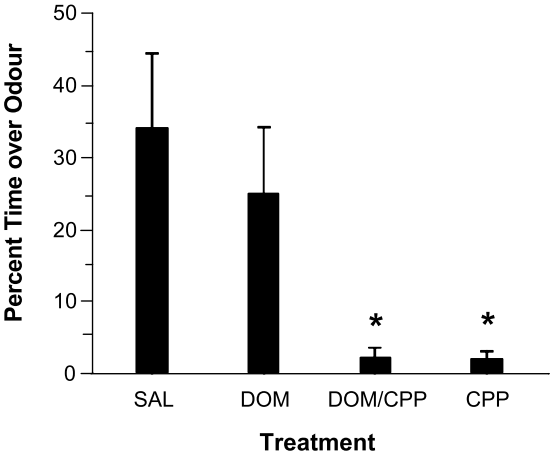

Fig. 1. Percent time spent over odour for groups rat pups tested on PND 9 (see text) following administration of saline (*SAL*), domoic acid (*DOM*), CPP or DOM in combination with CPP on PND 8 (see text). Data from animals tested with either peppermint or almond is combined. N = 16–17 for each group. *indicates $p < 0.05$ compared to saline

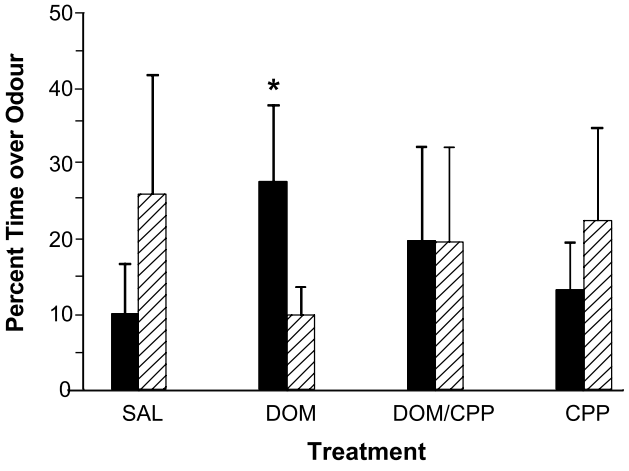

Fig. 2. Percent time spent over odour for groups of rats receiving daily injections of saline (*SAL*), domoic acid (*DOM*), CPP or DOM in combination with CPP from PND 8–13 and tested for odour preference on PND 13 using either peppermint (solid bars) or almond (hatched bars) (see text). N = 8–9 for each. *indicates $p < 0.05$ compared to saline

ent from saline. This difference was attributable to an effect of CPP on odour exploration and not to a non-specific effect of CPP on activity levels, since there was no significant difference in total grid crosses between groups ($F\ [3, 63] = 1.10$, $p > 0.05$).

When tested in the same paradigm on PND 13, rats treated with either saline or CPP spent almost 3-fold more time over the almond odour than their cohorts spent over the conditioning odour (peppermint), whereas this trend was completely reversed in the groups treated with domoic acid. DOM-treated rats spent significantly more time over peppermint than did saline-treated rats (one-tailed $U\ [9, 9] = 19.5$, $p < 0.05$) (Fig. 2). Furthermore, the effect of DOM was antagonized, at least in part, by co-administration of CPP (Fig. 2).

Discussion

We have previously reported that injection of very low doses of domoic acid during the second postnatal week in rats produces changes in simple associational learning as measured using conventional odour conditioning paradigms (Doucette et al., 2003). The use of doses well below those required to elicit any observable signs of toxicity (Doucette et al., 2000) makes it easier to attribute effects to selective activation of kainate receptors and is, perhaps, more consistent with understanding the ''physiologically relevant'' actions of kainate receptors in the CNS. While our previously published data (Doucette et al., 2003) demonstrated that on PND 13 DOM-treated rats spent significantly longer over the conditioning odour than did control rats, the testing paradigm used can be described as ''forced choice'' (i.e. the only choices the animal has are the conditioning odour or an alternate odour). The forced choice nature of the previous study inadvertently introduced a confound, whereby we could not determine definitively whether the DOM-treated rats ''preferred'' the conditioning odour or were avoiding the novel odour. The current study was designed to remove this confounding variable and to further explore the pharmacology of this effect.

Odours and scents are chemically complex, so it is entirely possible that neonatal rats may respond differently to odours or may be capable of discriminating bioactive molecules independent of odour. In the current study, time over odour data confirmed that there is no innate preference for either peppermint or almond in saline-treated neonatal rats. This means that any differences observed during odour preference testing can be reliably attributed to the conditioned response to the odour/drug combination. Testing on PND 9 also permitted the removal of the ''novelty'' confound, by exposing rat pups from all treatment conditions to the almond odour. These animals were then subsequently compared to groups that received identical handling but were re-exposed to peppermint on PND 9.

Additionally, data collected on PND 9 and presented in Fig. 1 presents compelling evidence that attention to odour is mediated almost exclusively by NMDA receptors in rats of this age. Rats treated with CPP at the time of odour conditioning spent significantly less time over odour than did pups treated with either saline or DOM in the absence of CPP (Fig. 1). NMDA receptors have been implicated previously in various tests of neonatal implicit learning in rats (Lincoln et al., 1988; Weldon et al., 1997; Mickley et al., 2000). The current results confirm that these findings can be generalized to neonatal odour conditioning paradigms when tested within the first two postnatal weeks.

To avoid the ''forced choice'' configuration used in our previously published study on 13 day old rats (Doucette et al., 2003), the current experiment compared groups of rats that were given a choice of ''odour'' versus ''no odour'' with a neutral zone in between to exaggerate the difference. Thus, rats tested using the conditioning odour (peppermint) could be clearly distinguished from rats tested using the alternate odour (almond). The finding that DOM-treated rats tested with peppermint spent significantly longer over the conditioning odour than did controls (Fig. 2), while showing no innate aversion to the almond odour (see above), confirms our previously published finding (Doucette et al., 2003). Moreover, the fact that this preference was completely opposite to the

behaviour of saline-treated rats (Fig. 2), and the fact that the paradigm removed novelty as a confounding variable, clearly confirms that injections of a very low dose of DOM appetitively reinforces a previous association with a conditioned stimulus (peppermint) in single trial learning on PND 8 (i.e. at low doses DOM can act as a nootropic agent in animals of this age).

The results presented in Fig. 2 also imply that concomitant administration of CPP antagonized, at least in part, the reinforcing properties of DOM at doses that did not alter preference when used alone (see above). These data indicate that NMDA receptors are critically involved in the response to DOM in this paradigm. NMDA receptor involvement is consistent with both the data obtained on PND 9 in the current study and with published data implicating NMDA receptor involvement in neonatal odour conditioning using other CS/US combinations. They are also consistent with literature on DOM toxicity that has reported involvement of NMDA receptor activation in DOM-mediated toxicity both *in vitro* (Novelli et al., 1992; Fernandez-Sanchez and Novelli, 1996; Berman and Murray, 1997) and *in vivo* (Tasker and Strain, 1998), suggesting that NMDA receptor activation, either directly or through the release of pre-synaptic glutamate, is an important component of the actions of DOM at both low (i.e. physiological) and high (i.e. toxicological) doses.

In conclusion, we have presented evidence that simple odour conditioning paradigms are a sensitive means of exploring the pharmacology of CNS development in neonatal rats. Moreover, the period of postnatal development between days 8 and 13 in the rat represents a particularly dynamic developmental window in which expression of this component of the behavioural phenotype appears to be dependent, at least in part, on both kainate and NMDA receptors.

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