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Genetic deletion of the adenosine A_{2A} receptor in mice reduces the changes in spinal cord NMDA receptor binding and glucose uptake caused by a nociceptive stimulus

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ABSTRACT

Mice lacking the adenosine A_{2A} receptor are less sensitive to nociceptive stimuli, and A_{2A} receptor antagonists have antinociceptive effects. We have previously shown a marked reduction in the behavioural responses to formalin injection in A_{2A} receptor knockout mice. This may be due to the presence of pronociceptive A_{2A} receptors on sensory nerves, and if so spinal cords from A_{2A} receptor knockout mice may have altered neurochemical responses to a nociceptive stimulus. We tested this hypothesis by studying two parameters known to change with spinal cord activity, NMDA glutamate receptor binding and [^{14}C]-2-deoxyglucose uptake, following intraplantar formalin injection in wild-type and A_{2A} receptor knockout mice. In naïve untreated A_{2A} knockout mice [^{14}C]-2-deoxyglucose uptake in all regions of the spinal cord was significantly lower compared to the wild-type, similar to the reduced NMDA receptor binding that we have previously observed. Following formalin treatment, there was an decrease in [^{3}H]-MK801 binding to NMDA receptors and an increase in [^{14}C]-2-deoxyglucose uptake in the spinal cords of wild-type mice, and these changes were significantly reduced in the A_{2A} knockout mice. In addition to altered behavioural responses, there are therefore corresponding reductions in spinal cord neurochemical changes induced by formalin in mice lacking adenosine A_{2A} receptors. These observations support the hypothesis that activation of A_{2A} receptors enhances nociceptive input into the spinal cord and suggests a possible role for A_{2A} antagonists as analgesics.

Adenosine can modulate pain pathways, and the nature of this action depends on the receptor subtypes involved. Adenosine acts via four G protein coupled receptors, A_1, A_{2A}, A_{2B}, and A_3. The A_1 and A_{2A} receptor have the highest affinity and are likely to be activated at physiological concentrations of adenosine, whereas the A_{2B} and A_3 receptors are more likely to play a role under pathological conditions such as ischaemia when high concentrations of adenosine are released [11,12]. Adenosine acts via A_1 receptors mainly in the spinal cord to inhibit nociception, whereas A_{2A} receptors have a pronociceptive effect which has been suggested to be due to an effect on peripheral nerve terminals [32]. In support of these opposing roles of the A_1 and A_{2A} receptors, A_1 knockout mice have enhanced nociceptive responses [19], whereas we and others have shown that A_{2A} knockout mice have reduced sensitivity to thermal nociceptive stimuli [3,14,24]. We have also shown a significant reduction in both phases of the nociceptive behaviour of A_{2A} knockout mice subjected to intraplantar formalin injection, and a marked antinociceptive effect of the selective A_{2A} antagonist 5-amino-7-(2-phenylethyl)-2-(2-*furyl*)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) in the same test [18]. Another study in mice also reported that an A_{2A}-selective antagonist injected locally into the paw caused reduction in both edema and pain responses to formalin [4].

In addition to these behavioural changes, we found a large reduction in the level of binding of [^{3}H](5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine ([^{3}H]-MK801) to NMDA glutamate receptors in the spinal cord of A_{2A} knockout mice, but no change in the binding of [^{3}H]substance P to NK1 receptors, showing that removal of the A_{2A} receptor has differential effects on the receptors for the two major transmitters released from primary afferent neurones in the spinal cord [18]. In A_{2A} receptor knockout mice there are also changes in spinal cord opioid receptor binding that are mirrored by changes in the antinociceptive potency of opioid receptor agonists [3].

The presence and function of A_{2A} receptors in the spinal cord are controversial. Conflicting results have been obtained from binding studies and autoradiography [3,7,8], and functional studies have reported some inconsistent effects of A_{2A} receptor ligands

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[1,5,15,16,25,28,36]. mRNA for the A_{2A} receptor has been reported to be expressed in the dorsal root ganglion but not in the spinal cord [20,21] suggesting that A_{2A} receptors could be present on the peripheral terminals of the sensory nerves but not to any significant extent in the spinal cord, as suggested by Sawynok [32]. More recent studies have however detected mRNA for the A_{2A} receptor in rat and mouse spinal cord [6,16], and it has been suggested that these may be on microglia as A_{2A} knockout mice have reduced responses (allodynia, hypoeralgesia and increased spinal cord microglia and astrocytes) in a neuropathic pain model thought to involve microglial activation [6]. In summary there seems to be evidence for the presence of A_{2A} receptors in the spinal cord but these receptors do not seem to exist at a density sufficient to be detected by autoradiography and their functional significance is not clear. The changes that we have observed in spinal cord receptor binding in the A_{2A} knockout mice are therefore not likely to be due to the absence of A_{2A} receptors in the spinal cord itself [3,18]. Instead the changes are likely to be related to the hypoalgesic phenotype of these mice, and may reflect reduced nociceptive input to the spinal cord due to loss of pronociceptive A_{2A} receptors on the peripheral terminals of sensory nerves.

To investigate further the hypothesis that the A_{2A} receptor is involved in pain pathways, and in particular the activation of sensory nerves which have their synapses in the spinal cord, we looked at changes in spinal cord neurochemistry following injection of formalin into the hind paw of wild-type and A_{2A} receptor knockout mice. Formalin is widely used as an acute nociceptive stimulus, with two distinct phases: the first phase (0–15 min) reflecting mainly direct stimulation of sensory nerves, with the second phase (15–60 min) reflecting an inflammatory component [10,33]. As glutamate is the major neurotransmitter released by primary afferent neurones [27], we measured binding to ionotropic NMDA glutamate receptors in the spinal cord as changes in nerve firing are likely to be followed by changes in receptor binding and because changes in the expression and phosphorylation state of NMDA receptor subunits in the spinal cord have been reported in the spinal cord after treatment with formalin [13,22]. We have also looked at the *in vivo* uptake of [^{14}C]-deoxyglucose in the spinal cords of these mice as a marker of neuronal activity [34], because previous studies using this method have shown an increase in the uptake of [^{14}C]-2-deoxyglucose in both phases of the formalin test in both rats and mice [2,30,31].

Animals: Wild-type and A_{2A} knockout age-matched male mice on a CD1 background [24] aged 8–12 weeks were bred from heterozygotes and genotyped at weaning. All experiments described followed protocols agreed by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK.

Formalin treatment: Mice were lightly restrained before receiving a single 20 μl injection of 5% formalin solution subcutaneously into the ventral, plantar surface of the left hind paw. Mice were assigned to one of two groups designated 'early' or 'late', corresponding to the two phases of the response to formalin. Mice assigned to the 'early' group were killed 15 min after formalin injection, whereas mice designated to the 'late' group were killed 60 min following formalin injection. The spinal cords were dissected out, frozen in isopentane at −25 °C and stored at −80 °C until required.

For the study of [^{14}C]-2-deoxyglucose uptake mice designated to the 'early' group were restrained in a plastic cylinder, their tails warmed under a heat lamp and 3700 kBq/kg [^{14}C]-2-deoxyglucose dissolved in sterile saline (stock concentration of 3700 kBq/ml) was injected intravenously via the tail vein. After 5 min they were treated with formalin, then returned to the observation chamber for a further 15 min before being killed. "Late" mice were treated with formalin 15 min before they were injected with 3700 kBq/kg [^{14}C]-2-deoxyglucose. They were then returned to the observation chamber for a further 45 min before being killed. Control mice for each group followed the same treatment protocol as "early" or "late" mice but received no formalin injection.

Spinal cord receptor autoradiography: Sections (20 μm) were cut from all four anatomical regions of the spinal cord (cervical, thoracic, lumbar and sacral) using a crysostat (Microm 505E, Zeiss, UK) maintained at −20 °C and thaw-mounted onto gelatine-coated slides. Adjacent sections were cut for determination of total binding and non-specific binding (NSB). Slides with tissue sections were placed into storage boxes containing desiccant (Drierite) for a period of 2 h at 4 °C before being frozen at −20 °C until used.

Binding to NMDA receptors was determined using [^{3}H]-MK801 as previously described [18]. Sections were pre-incubated in 50 mM Tris buffer (Trizma) at pH 7.4 containing 1 μM glutamate, 1 μM glycine and 1 μM spermidine for 20 min at room temperature. Total binding was determined by incubating in the same buffer with 70 nM [^{3}H]-MK801 for 1 h at 4 °C, and NSB was determined by the addition of 1 mM unlabelled MK801. Sections were washed for a total of 60 s in three changes of ice-cold, briefly rinsed in distilled water and dried in a stream of cool air.

Slides were placed into autoradiography cassettes and apposed to [^{3}H]-Hyperfilm for 3 weeks alongside [^{3}H]-microscale standards (4048 –3.74 Bq/mg). The resultant autoradiograms were developed in Develex for 5 min, washed in distilled water for 30 s and fixed in Amfix fixative for 4 min, washed for 30 min in distilled water then air-dried.

Autoradiographic assessment of [^{14}C]-2-deoxyglucose uptake: Sections (20 μm) were cut, mounted and dried as above, then the slides were apposed for three weeks to Kodak MR-1 film alongside [^{14}C]-microscale standards (31.89 –1.11 kBq/g). The resultant autoradiograms were developed in Kodak D-19 for 75 s, washed in distilled water containing acetic acid for 30 s and fixed in Kodak rapid fixer for 3 min, washed in distilled water for 30 min and then air-dried.

Analysis of autoradiographic images: Quantitative analysis was performed using an MCID imaging system (Imaging Research, Canada). For each region of spinal cord examined, at least three sections were used for quantification. All anatomical areas of the spinal cord were analysed by free-hand drawing and referenced to the rat atlas of Paxinos and Watson [29]. For [^{3}H]-MK801 binding, measurements were taken from laminae I-II, III-VI, VII-IX and X on both left and right sides for each section analysed, therefore representing a duplicate determination in each section except for lamina X where only one measurement was taken. Although data were originally obtained from ipsilateral and contralateral sides of the spinal cord there were no significant differences observed between the two sides so data were pooled. There were also no differences between the different laminae after either the early or the late phase of formalin treatment in the binding of [^{3}H]-MK801, so the data were pooled for clarity and to provide a more robust comparison between the genotypes. For [^{14}C]-2-deoxyglucose uptake the images were not so well resolved but measurements were taken from laminae I-VI, VII-IX and X for the naïve mice. As there were no differences between the laminae in these mice and inspection of the films for the treated mice did not indicate any obvious visual differences, for the treated mice measurements were taken from the whole spinal cord section to improve accuracy and provide a more robust comparison between the genotypes. Radioligand binding was quantified by reference to the [^{3}H]-microscale standards and expressed as fmol/mg tissue. [^{14}C]-2-deoxyglucose uptake was quantified by reference to the [^{14}C]-microscale standards and expressed as kBq/g.

Data analysis and statistical procedures: Because of the different basal values observed between naïve wild-type and adenosine A_{2A} receptor knockout mice, data were transformed to generate values as a % of control. Control values for [^{3}H]-MK801 binding were