ORIGINAL ARTICLE

Intraperitoneal injection of saline modulates hippocampal brain receptor complex levels but does not impair performance in the Morris Water Maze

**Ajinkya Sase · Deeba Khan · Harald Höger ·
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Abstract The involvement of the hippocampus in pain has been demonstrated but key players, i.e. the major brain receptors have not been shown to be modulated by pain. It was therefore the aim of the study to show the concerted action and pattern of brain receptor complex levels in a non-invasive model of moderate pain. C57BL/6J mice were divided into four groups of 14 animals each: trained injected, trained non-injected, yoked injected and yoked non-injected. Animals were tested in the open field and the elevated plus maze for behavioural evaluation and cognitive functions were tested using the Morris Water Maze. Hippocampi were taken 6 h following sacrification. Membrane proteins were prepared by ultracentrifugation and run on blue native gels to keep the native state, blotted to membranes and western blotting was carried out using the primary antibodies against serotonin receptor 5HT1A, muscarinic acetylcholine receptor M1 (mAChR-M1), nicotinic acetylcholine receptor alpha7 (nAChR-alpha7), glutamate (AMPA) receptor (GluR1) and neurokinin receptor 1 (NK-1). There was no difference between performance in behaviour or in the MWM between groups. Brain receptor level changes involved all receptors given above. Pain affected mAChR-M1, GluR1 and NK-1 complex levels when yoked-injected were compared with

yoked non-injected animals. Memory mechanisms affected mAChR-M1 complex levels when trained non-injected animals were compared with yoked non-injected controls. Taken together, the neurochemical basis for testing receptor agonists/antagonists on the role of pain and the hippocampus was generated that may be useful for interpretations of the role of this complex area in moderate pain.

Keywords Intraperitoneal injection · Pain · Morris Water Maze · Serotonin · Muscarinic receptor · Nicotinergic receptor

Introduction

Pain processing in rodents is a highly complex process inducing varied responses in the brain. A series of studies have been carried out to understand the roles of various brain receptors underlying nociceptive transmission with conflicting results: Serotonin receptors (5-HTRs) have been implicated in pain responses and Granados-Soto et al. (2010) demonstrated that peripheral 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F receptor subtypes modulate antinociceptive responses by formalin-induced pain in rats and observed significantly reduced formalin-induced flinching upon administration of 5-HTR agonists R(+)-UH-301 (5-HT1A), CGS-12066A (5-HT1B), GR46611 (5-HT1B/1D), BRL54443 (5-HT1E/1F) and LY344864 (5-HT1F). In order to verify the antinociceptive effect of these agonists, specific 5-HT antagonists WAY-100635 (5-HT1A), GR5562 (5-HT1B/D), SB224289 (5-HT1B) were administered and a decrease in the antinociceptive effects of the agonists was observed, thus confirming a role of 5-HTRs in nociception. Martel et al. (2009) also demonstrated involvement of 5HT1A receptors in nociceptive

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pain tests on rats by using the specific 5-HT1A receptor agonist xaliproden (SR57746A). Recent evidence has implicated the muscarinic receptors in nociception: Cai et al. (2009) have shown that M2 and M4 muscarinic acetylcholine receptors (mAChRs) are involved in nociceptive regulation by using small-interference RNA (siR-NA), specifically targeting mAChR subtypes in rats. Honda et al. (2002) observed inhibition of pain responses in rats using M_3 receptor antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP). Cannabinoid receptor CB1 mRNA was demonstrated to be expressed throughout the adult mouse brain and in significant but low levels in the hippocampus (Marsicano and Lutz 1999). There have been various studies to show that the CB1 receptor is involved in attenuating pain. Using a non-selective cannabinoid agonist CP 55,940 in CB1 receptor knockout mice ($CB1^{-/-}$), Sain et al. (2009) demonstrated the importance of CB1 receptors in mouse neuropathic pain. Furthermore, Kinsey et al. (2009) showed the importance of CB1 receptors in analgesia by pretreatment with CB1 and CB2 receptor antagonists and observed reversal of pain that had been inhibited in C57BL/6J mice by chemically inhibiting principal enzymes responsible for endogenous cannabinoids. Zhao et al. (2009) evoked a nociceptive response in rats by either bee venom or formalin administration and observed an increased number of detectable field excitatory postsynaptic potentials (mediated by ionotropic glutamate receptors) in the dentate gyrus and cornu ammonis 1 (CA1) regions of hippocampal slices of rats. Various studies have shown an analgesic effect upon administration of antagonists acting on ionotropic glutamate receptors in the hippocampus, linking nociceptive responses to changes in hippocampal NMDA and AMPA receptors. In McKenna and Melzack (2001) successfully demonstrated that blocking NMDA receptors in rats by 2-amino-5-phosphonovalerate (AP5) administration significantly reduces pain behaviours of the formalin pain tests. In 2006 Duric and colleagues addressed the role of NK1 receptors in analgesic responses in the hippocampus and spinal cord using indomethacin (a nonsteroidal anti-inflammatory drug) and imipramine (a tricyclic antidepressant drug) and reported that although nociceptive drug treatment reduced pain sensory activation in the spinal cord, it was insufficient to prevent the impact of pain on the hippocampus. In addition the alpha7 nicotinic receptor (nAChR) has been implicated in neuropathic pain as well. Pacini et al. (2010) demonstrated the importance of nAChR-alpha7 by reducing the analgesic effects of methyllicaconitine using the nAChR-alpha7 agonist PNU-282987. Therefore, there are specific receptors involved in nociceptive responses in the hippocampus.

There is much literature to support that stress plays a major role in the hippocampus and affects synaptic plasticity and memory formation (Kim et al. 2006; McEwen and Magarinos 2001; Janitzky et al. 2011). Furthermore, in addition to its well-documented roles in learning and memory formation, there is substantial evidence linking the hippocampus to pain processing. Al Amin et al. (2004) showed the importance of proper hippocampal development in pain processing by lesioning the ventral hippocampus in neonates and observing hyperalgesia in hot plate and paw pressure tests at puberty, which indicated that a ventral hippocampal lesion partially alleviates pain processing. By observing a decrease in Fos-positive cells along the length of the hippocampus upon injection with formalin, Khanna et al. (2004) proved that nociceptive information is processed and distributed along the hippocampus and that the ventral CA1 region plays a role in pain responses. In a related study, Kazi et al. (2007) tested two peptides which may have roles in nociception and other behavioural paradigms and detected that c-Fos expression increased in the CA2 and CA3 regions of the hippocampus upon administration of N/OFQ (a possible endogenous ligand for the orphan opioid receptor like receptor which causes hyperalgesia) and attenuated upon co-administration of its antagonist nocistatin. Teather et al. (2006) demonstrated involvement of hippocampal plasma membrane platelet activating factor (PAF) receptors in pain processing by using a specific hippocampal membrane PAF antagonist in rats and observed a decrease in late phase nociceptive responses. Receptors in the dentate gyrus and CA1 regions of the hippocampus have also been attributed to pain processing. Soleimannejad et al. (2006) demonstrated the importance of 5HT2A/2C receptors, specifically in the dentate gyrus and CA1 regions of the hippocampus in pain processing by chemically blocking the 5HT2A/2c receptors using ritanserin and observing a significant decrease in the nociceptive responses of rats. The following year, Soleimannejad et al. (2007) reported the importance of NMDA-sensitive mechanisms involved in pain processing in the dentate gyrus and CA1 regions by making use of the NMDA receptor antagonists AP5 and MK-801; a significant decrease in pain-related behaviours when the antagonists were administered in a dose-dependant manner was shown. All these results taken together implicate the hippocampus in acute pain-processing and suggest receptor targets for analgesic drug treatment.

Due to consistently good performance in the Morris Water Maze task (MWM), Multiple T-maze (MTM), Barnes maze and contextual fear conditioning, the C57BL/6J strain is widely accepted as a suitable mouse model for hippocampus-dependent learning and memory. The aims of the study were to find out whether moderate pain induced by intraperitoneal injections would interfere with performance in the MWM and if major brain receptors that have been linked to pain were modulated in the hippocampus.

The study shows a pattern of native brain receptor complexes revealing the concerted action in the mouse hippocampus following induction of moderate pain.

Materials and methods

Animals

Male C57BL/6 mice were used for the studies. Mice used were aged between 10 and 14 weeks, because at this age development is complete and ageing processes are far from being started. All the mice were purchased from JANVIER SAS Laboratories (France) and maintained in cages made of Makrolon and filled with autoclaved woodchips in the Core Unit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna. Autoclaved standard rodent diet (Altromin, Germany) and water in bottles were available ad libitum. The room was illuminated with artificial light at an intensity of about 200 lx in 2 m from 5 am to 7 pm. Behavioural tests were performed between 8 am and 1 pm. Animals were divided into 4 groups. Each group contained 14 animals. Two groups were trained and other two groups were used as yoked controls. Controls were grouped into two as injected with saline and non-injected. Injected groups were given intraperitoneally (IP) isotonic saline 0.5 mL/kg of body mass, 30 min before the start of the experiment.

All procedures were carried out according to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by Federal Ministry of Education, Science and Culture, Austria (BMWF-66.009/0240-II/106/2009). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Behavioural studies

Open field (OF)

Mice were observed with a Video monitoring system (video camcorder: 1/3″ SSAM HR EX VIEW HAD coupled to computational tracking system: TiBeSplit) consisting of a Video camcorder coupled to a computational tracking system in an arena (40 cm × 40 cm long; with 70 cm high walls) for 10 min. Mice were placed into the centre and following parameters were measured: (a) frequency of sniffing at the wall, (b) rearing, (c) crossing the centre, (d) entries into the centre, (e) time spent in the centre of the arena, (f) resting time and (g) average speed (Weitzdoerfer et al. 2004).

Elevated plus maze (EPM)

Mice were observed for anxiety-like behaviour. The maze consisted of 4 arms (each 30 cm long and 5 cm wide) fixed to the height of 54 cm, and the arms were interconnected by a 5 cm × 5 cm wide central area. Two arms had 15-cm-high side and end walls. Mice were observed for 5 min with video camcorder coupled to a computational tracking system in an arena. The mice were placed in the central area, head pointing away from the box. Following parameters were recorded: (a) the time spent in open arm and closed arm, (b) number of entries into the open arm and closed arm, (c) pathlength in open and closed arm. An entry was defined as having the mouse placed on all four legs into the box (Weitzdoerfer et al. 2004).

Cognitive studies

Morris water maze (MWM)

The MWM consists of a circular pool (122 cm diameter, wall depth 76 cm) in which mice were trained to escape from water by swimming to a hidden platform (1.5 cm beneath water surface) whose location can be only identified using distal extra-maze cues attached to the room walls. Water temperature was maintained at $21 \pm 1°C$.

The pool was divided into four quadrants by a computerized tracking/image analyzer system (video camcorder: 1/3″ SSAM HR EX VIEW HAD coupled to computational tracking system: TiBeSplit). The platform was placed in the middle of the SE quadrant and remained at the same position during the training experiment.

The spatial acquisition phase consisted of four training trials per day and four training days. Mice were released with their heads facing the pool wall from the four compass locations (NE, NW, SW, and SE randomly) and allowed to swim and search for the platform for 120 s. If mice did not locate the platform after 120 s, animals were manually placed on the platform and allowed to remain on it for 30 s. Each animal was then returned to its cage for 10 min before its next trial. On the first training day, mice were given an acclimatization training session in the water maze; mice were placed on the hidden platform, were allowed to swim for 30 s, and were guided subsequently back to the platform, climbing onto the platform. The latency and path length to reach the hidden platform and average swim speed were recorded.

On day 5 animals received a probe trial, in which the platform was removed. The mouse was released from NE start point and allowed to swim freely for 60 s. The time spent in each quadrant was calculated.

Yoked controls were placed in the water maze to swim the same amount of time as their trained partners, but

without a platform being present to climb onto. Animals were exposed to the same spatial cues, but without an escape platform; therefore, mice did not develop an association between the extra-maze cues and the location of the platform. Yoked controls were grouped into two as injected with saline and non-injected. Yoked controls injected with saline were used to rule out possible differences in protein expression by saline effect, whereas yoked non-injected controls ruled out possible differences in protein expression due to stress and physical effort (swimming) produced in the maze.

Six hours after completion of probe trial, mice were deeply anaesthetized with CO_2 and killed by neck dislocation. Hippocampi were rapidly dissected and stored at –80°C for biochemical analysis.

Sample preparation

Total membrane fraction

28 hippocampi of trained and yoked mice each (total $n = 56$) were homogenized in ice-cold homogenization buffer [10 mM HEPES, pH 7.5, 300 mM sucrose, one complete protease inhibitor tablet (Roche Molecular Biochemicals, Mannheim, Germany) per 50 mL] by Ultra-Turrax® (IKA, Staufen, Germany). The homogenate was centrifuged for 10 min at $1,000 \times g$ and the pellet was discarded. The supernatant was centrifuged at $50,000 \times g$ for 30 min in an ultracentrifuge (Beckman Coulter Optima® L-90 K). Subsequently, the pellet was homogenized in 5 mL washing buffer (homogenization buffer without sucrose), kept on ice for 30 min and centrifuged at $50,000 \times g$ for 30 min. All the individual 56 samples were used for the gel experiments.

Sucrose gradient ultracentrifugation

The plasma membrane purification procedures from the pellet were carried out as described previously, with slight modifications (Kang et al. 2008; Heo and Lubec 2010). Sucrose density gradient centrifugation solutions of 700 μL each of 69, 54, 45, 41, and 37% (w/v) were formed. Membrane pellets in 500 μL were resuspended in

homogenization buffer, layered on top of the tubes that were filled with homogenization buffer. Samples were ultracentrifuged at 4°C at $70,000 \times g$ for 3 h. After centrifugation, the 41% fraction from the sucrose interface was collected, diluted 10 times with homogenization buffer and then ultracentrifuged at 4°C at $100,000 \times g$ for 30 min. After discarding the supernatant, the pellet was stored at –80°C until use.

Extraction of membrane receptor protein

Membrane pellets from the 41% sucrose gradient ultracentrifugation fraction were solubilized in extraction buffer [1.5 M 6-aminocaproic acid, 300 mM Bis–Tris, pH 7.0] and 10% Triton X-100 [stock solution was added at a ratio of 1:4 to achieve final 2% Triton X-100 concentration] with vortexing every 10 min for 1 h. Following solubilization, samples were cleared by centrifugation at $20,000 \times g$ for 60 min at 4°C. The protein content was estimated using the BCA protein assay kit (Pierce, Rockford, IL, USA). Extracted membrane receptor proteins were then aliquoted and stored at –80°C till use.

Blue native PAGE

50 μg of prepared samples was loaded for muscarinic acetylcholine receptor subtype M1 (mAChR-M1), neuronal acetylcholine receptor subtype alpha 7 (nAChR-alpha7) and neurokinin (NK1) receptors. 30 μg of prepared sample was loaded for serotonin 1A (5HT1A) and Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor subtype Glu1 (GluR1). 16 μL BN PAGE loading buffer [5% (w/v) Coomassie G250 in 750 mM 6-aminocaproic acid] was mixed with 100 μL of the prepared sample and loaded onto the gel. BN-PAGE was performed in a PRO-TEAN II xi Cell (BioRad, Germany) using 4% stacking and 5–18% separating gel.

The BN-PAGE gel buffer contained 500 mM 6-aminocaproic acid, 50 mM Bis–Tris, pH 7.0; the cathode buffer 50 mM Tricine, 15 mM Bis–Tris, 0.05% (w/v) Coomassie G250, pH 7.0; and the anode buffer 50 mM Bis–Tris, pH 7.0. The voltage was set to 50 V for 1 h, 75 V for 2 h, 100 V for 2 h and was increased sequentially to 400 V

Table 1 List of antibodies and dilutions used

Antibody	Species	Company and catalogue number	Dilution used
5HT1A receptor	Rabbit polyclonal	GenScript, USA (Heo and Lubec 2011)	1/20000
mAChR-M1 receptor	Rabbit polyclonal	Abcam-AB75178	1/5000
nAChR-alpha7 receptor	Rabbit polyclonal	Abcam-AB23832	1/3000
GluR1 receptor	Rabbit polyclonal	Abcam-AB31232	1/20000
NK-1 receptor	Rabbit polyclonal	Abcam-AB75516	1/3000

Fig. 1 Results of the open field. Mice injected with saline showed no significant differences versus non-injected mice
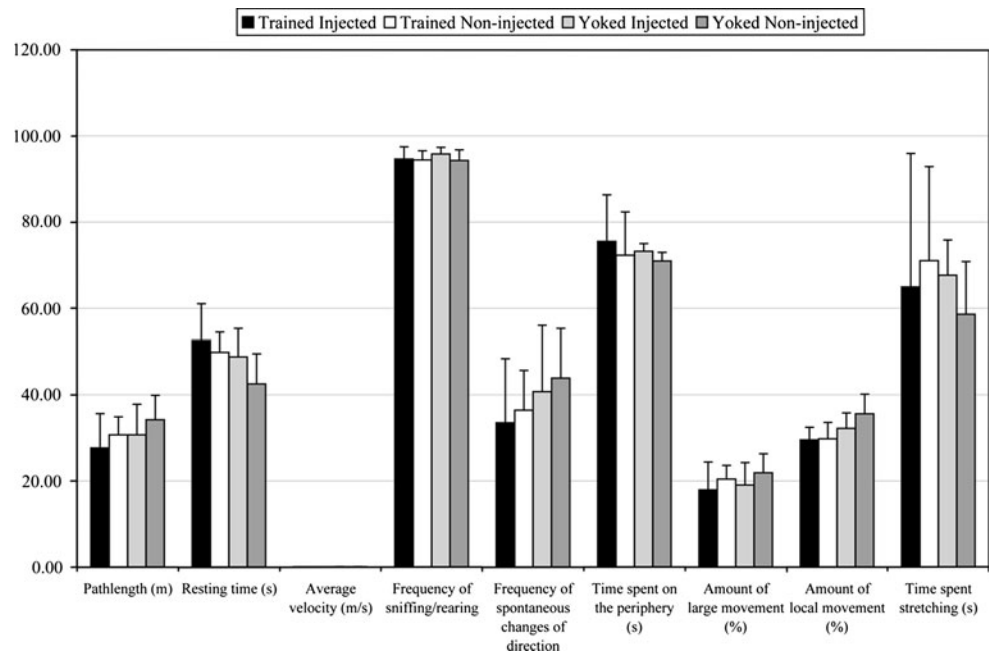
(maximum current 15 mA/gel, maximum voltage 500 V) until the dye front reached the bottom of the gel (Kang et al. 2008) Native high-molecular-mass markers were obtained from Invitrogen (Carlsbad, CA, USA).

Western blotting

Proteins separated on the gel were transferred onto PVDF membranes. After blocking with 5% non-fat dry milk in 0.1% TBST (100 mM Tris–HCl, 150 mM NaCl, pH 7.5, 0.1% Tween 20) for 1 h at 21°C, membranes were incubated overnight at 4°C with gentle agitation using diluted primary antibodies given in Table 1.

After six times washing with 0.1% TBST, membranes were incubated with horseradish peroxidase-conjugated Goat polyclonal Secondary Antibody to Rabbit IgG (AB6722, Abcam, Cambridge, UK) and then again washed with 0.1% TBST. Membranes were developed with the ECL plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densities of immunoreactive bands were measured by the Image J software program (http://rsb.info.nih.gov/ij/).

Statistical analysis

In the MWM the treatment effect in time spent in the target quadrant during the retention phase was analysed by ANOVA with subsequent unpaired Student's t test. For the acquisition phase in the MWM the treatment effect in latency to reach the platform was investigated for each group. Pair-wise comparisons of groups were done by ANOVA. A probability level of $P < 0.05$ was considered

as statistically significant. All calculations were performed using SPSS version 14.0 (SPSS, Inc., Chicago, IL). In the proteomic part of the study, statistical analysis to reveal between-group differences was performed by ANOVA followed by unpaired Student's t test. Pearson and
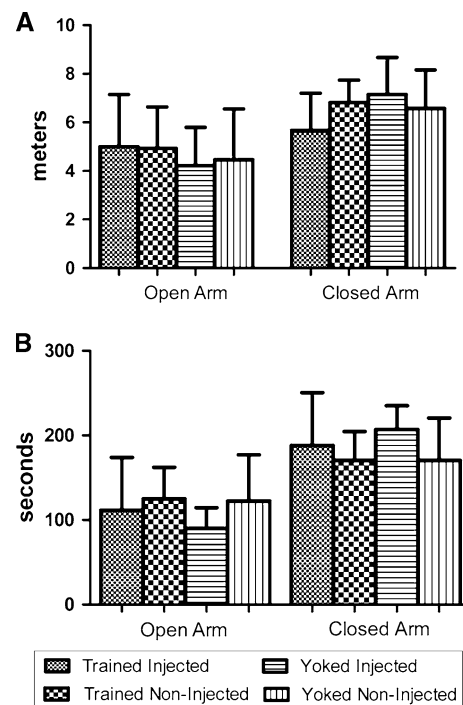

Fig. 2 Results of the elevated plus maze. **a** Pathlength of injected and non-injected animals in each arm is shown. **b** Time spent in each arm by injected and non-injected mice: no significant difference was observed between injected and non-injected animals

Fig. 3 a The latency to reach
the platform during training
days in the MWM is shown. A
clear learning curve was
obtained for trained injected and
trained non-injected mice. **b,
c** Pathlength and speed are
comparable between groups
during the training days.
b Pathlength decreases with
increasing training days.
c Average speed increases with
increasing training days. **d,
e** Means and SD from the probe
trial for the time spent in each
quadrant: **d** trained injected
mice, **e** trained non-injected
mice. *$P < 0.05$,
***$P < 0.001$. **f** Time spent in
target quadrant by trained
injected and trained non-
injected animals. No significant
difference was observed
between injected and non-
injected animals, ns = P value
not significant
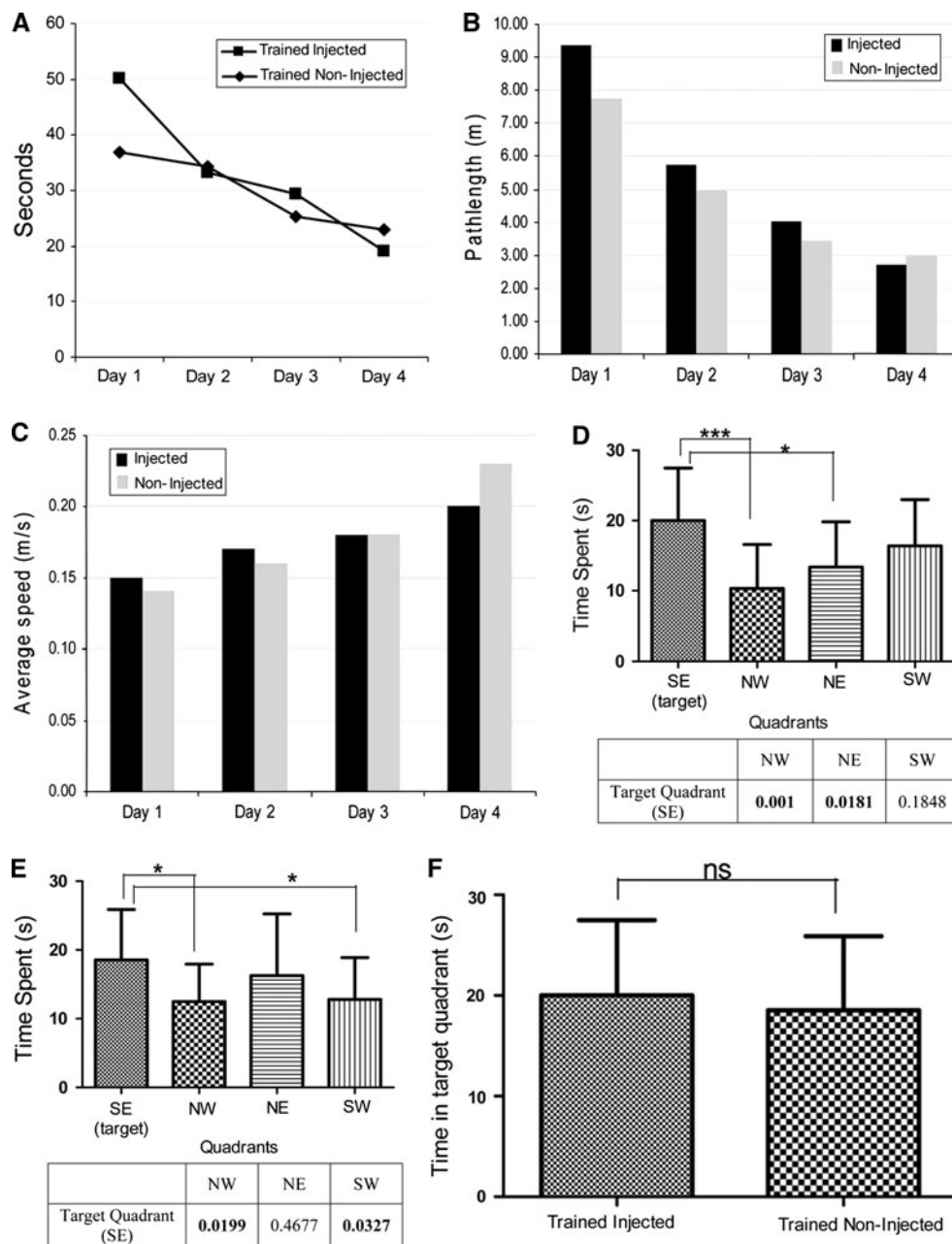
Spearman correlations were calculated using the SPSS
version given above.

Results and discussion

The major finding of the current study is that repeated
intraperitoral (IP) injection to C57BL/6J mice leads to
brain receptor changes but does not influence spatial
learning and memory. Behaviours in the open field as well
as in the EPM were comparable between groups (Figs. 1,
2). Both, retrieval and the pain from peritoneal injections
modulated brain receptor complex levels.

In the MWM latency significantly decreased along the
four training days indicating that trained groups learned the
task (Fig. 3a). In the acquisition phase speed and path
lengths were comparable between days of training (Fig. 3b,
c). At the probe trial on day 5, trained mice, injected or
non-injected, showed successful retrieval expressed as time
spent in the target quadrant (Fig. 3d, e). Times spent in the
target quadrant in trained injected versus trained non-
injected showed no significant difference (Fig. 3f).

These results are proposing that intraperitoneal injec-
tions widely in use in pharmacological studies do not
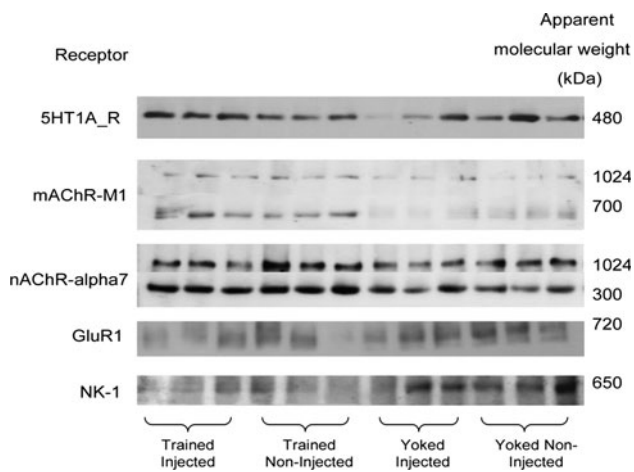change exploratory and anxiety-related behaviour (OF,
EPM).

Fig. 4 Fourteen animals were used for immunoblotting and bands, representative for the individual groups are shown

A series of brain receptors, including serotonin, muscarinic, AMPA, nicotinic alpha 7, NMDA and NK1 receptors, have been shown to be involved in pain, although most of these were not tested in the hippocampus and were not determined as receptor complexes, but indeed, it is the complexes that are functional receptor units.

Representative blue native (BN)-western blot images are provided in Fig. 4 along with apparent molecular weights based upon comparison with native gel marker proteins and the Table 2 shows statistical results for 14 trained injected, 14 trained non-injected, 14 yoked injected and 14 yoked non-injected controls. The antibodies against five different receptors recognized a single band as shown in Fig. 4, except for the nAChR-alpha7 and mAChR-M1 that showed two bands each. Table 2 shows means and standard deviations of receptor levels.

As shown in Fig. 5a, there was a trend ($P = 0.0259$) to higher levels when trained injected mice were compared to yoked injected mice, thus being in agreement with previous results of 5-HT1A-R complex levels obtained in another

land maze (Heo et al. 2011). For this receptor no effect by the intraperitoneal injection (IP) in trained or yoked groups could be shown. In all experiments the level of significance was set at $P < 0.01$ and P levels lower than 0.05 were considered a trend. The 5-HT1A-R levels therefore may not play a role for pain or stress by the IP in the hippocampus.

Figure 5b shows the mAChR-M1 high-molecular-weight (HMW) complex levels at a higher apparent molecular weight and this band showed a trend of difference when trained injected were compared with trained non-injected animals. The statistical difference between trained injected and yoked injected animals points to a learning effect. Only a trend in changes of receptor levels between yoked injected and yoked non-injected animals was observed, thus not providing sufficient evidence for a pain effect.

Figure 5c shows the effects of IP on the mAChR-M1 low-molecular-weight (LMW) complex levels: A trend to lower levels was observed comparing trained-injected versus yoked injected ($P = 0.012$), probably indicating involvement of this receptor in memory performance and indeed, this receptor has already been proposed to play a role in memory (Cai et al. 2009). IP had a strong effect on mAChR-M1 complex levels increasing receptor complex levels in trained injected mice, and this effect was also seen comparing yoked injected with yoked non-injected mice. It is proposed that memory as well as IP are modulating hippocampal mAChR-M1 complex levels. So far only M2,3,4 levels have been shown to be involved in pain (Cai et al. 2009; Honda et al. 2002). The biological and pharmacological relevance of this finding remain open as well as the specific role of the hippocampus in pain/stress, although hippocampal functions per se may be modulated by receptor level changes.
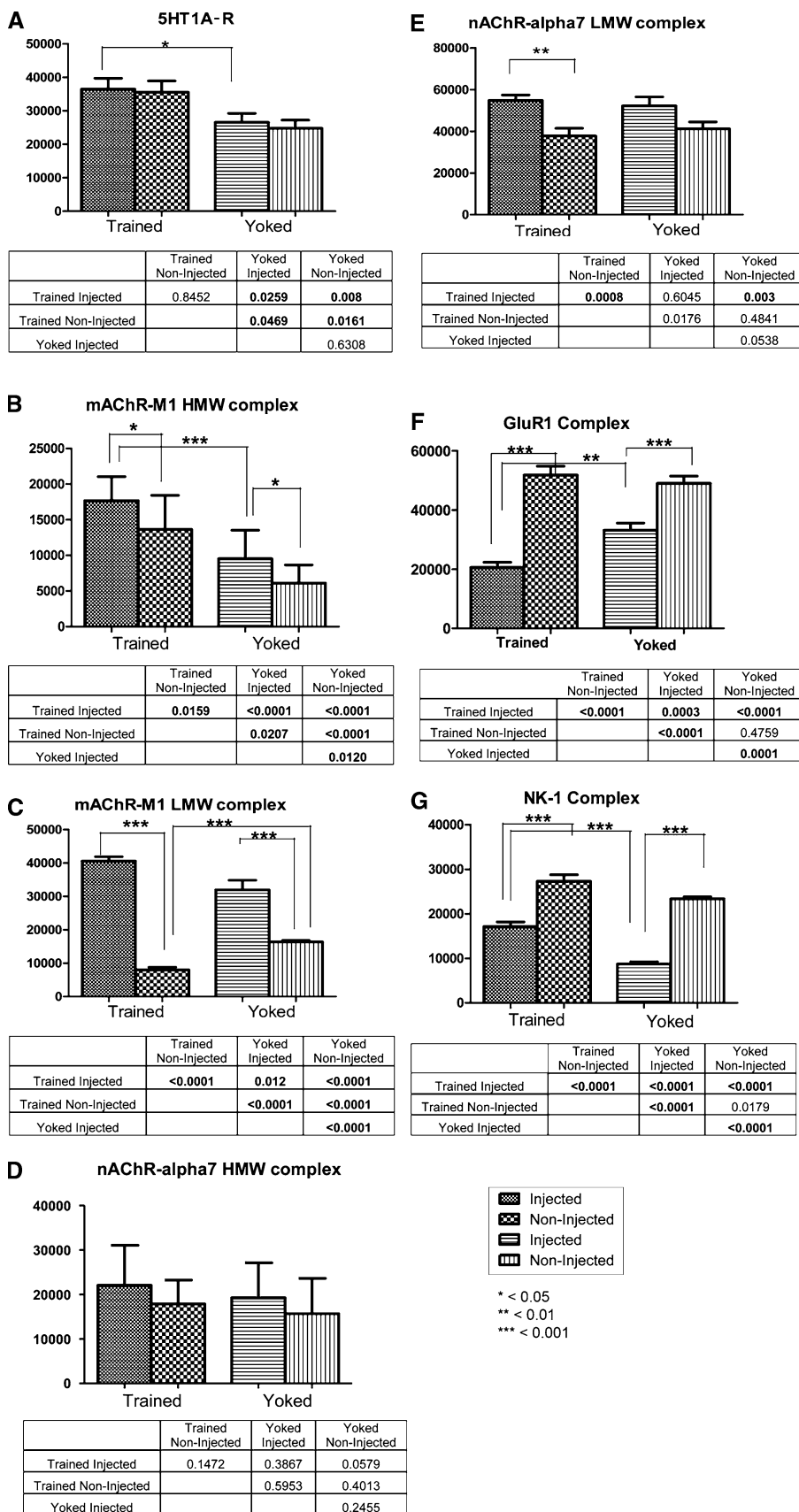
Figure 5d shows that the band at the higher apparent molecular weight of the nAChR-alpha7 high-molecular-weight complex levels did not show any statistical differences and this finding was used as loading control, while

Table 2 Densitometric values (mean $\pm$ standard deviation) obtained for each group of animals

Receptor		Mean $\pm$ SD ($n = 14$)			
		Trained injected	Trained non-injected	Yoked injected	Yoked non-injected
5HT1A		36,484 $\pm$ 12,139	35,559 $\pm$ 12,676	26,555 $\pm$ 10,000	24,788 $\pm$ 9,203
mAChR-M1	HMW	17,682 $\pm$ 3,379	13,645 $\pm$ 4,781	9,540 $\pm$ 4,001	6,122 $\pm$ 2,529
	LMW	40,594 $\pm$ 4,815	8,041 $\pm$ 2,833	31,968 $\pm$ 10,788	16,399 $\pm$ 1,488
nAChR-alpha7	HMW	22,052 $\pm$ 9,013	17,869 $\pm$ 5,343	19,236 $\pm$ 7,872	15,691 $\pm$ 7,915
	LMW	54,805 $\pm$ 9,719	37,782 $\pm$ 13,671	52,156 $\pm$ 16,212	41,242 $\pm$ 12,073
GluR1		20,555 $\pm$ 6,616	51,774 $\pm$ 11,176	33,135 $\pm$ 9,191	48,985 $\pm$ 9,120
NK-1		17,119 $\pm$ 3,927	27,273 $\pm$ 5,538	8,706 $\pm$ 1,910	23,358 $\pm$ 1,702

HMW high molecular weight, *LMW* low molecular weight

Fig. 5 Hippocampal expression levels of **a** 5HT1A-R; **b** mAChR-M1 (HMW); **c** mAChR-M1 (LMW); **d** nAChR-alpha7 (HMW); **e** nAChR-alpha7 (LMW); **f** GluR1 and **g** NK-1 including *P* values for individual receptor levels in all groups

A 5HT1A-R

	Trained Non-Injected	Yoked Injected	Yoked Non-Injected
Trained Injected	0.8452	**0.0259**	**0.008**
Trained Non-Injected		**0.0469**	**0.0161**
Yoked Injected			0.6308

B mAChR-M1 HMW complex

	Trained Non-Injected	Yoked Injected	Yoked Non-Injected
Trained Injected	**0.0159**	**<0.0001**	**<0.0001**
Trained Non-Injected		**0.0207**	**<0.0001**
Yoked Injected			**0.0120**

C mAChR-M1 LMW complex

	Trained Non-Injected	Yoked Injected	Yoked Non-Injected
Trained Injected	**<0.0001**	**0.012**	**<0.0001**
Trained Non-Injected		**<0.0001**	**<0.0001**
Yoked Injected			**<0.0001**

D nAChR-alpha7 HMW complex

	Trained Non-Injected	Yoked Injected	Yoked Non-Injected
Trained Injected	0.1472	0.3867	0.0579
Trained Non-Injected		0.5953	0.4013
Yoked Injected			0.2455

E nAChR-alpha7 LMW complex

	Trained Non-Injected	Yoked Injected	Yoked Non-Injected
Trained Injected	**0.0008**	0.6045	**0.003**
Trained Non-Injected		0.0176	0.4841
Yoked Injected			0.0538

F GluR1 Complex

	Trained Non-Injected	Yoked Injected	Yoked Non-Injected
Trained Injected	**<0.0001**	**0.0003**	**<0.0001**
Trained Non-Injected		**<0.0001**	0.4759
Yoked Injected			**0.0001**

G NK-1 Complex

	Trained Non-Injected	Yoked Injected	Yoked Non-Injected
Trained Injected	**<0.0001**	**<0.0001**	**<0.0001**
Trained Non-Injected		**<0.0001**	0.0179
Yoked Injected			**<0.0001**

Injected
Non-Injected
Injected
Non-Injected

* < 0.05
** < 0.01
*** < 0.001

nAChR-alpha7 low-molecular-weight complex levels were higher in trained injected mice than in trained non-injected subjects. This may indicate that only training combined with IP led to receptor level changes. Both receptors have been shown to interact/counteract in the hippocampus which is not supported herein (Shen et al. 2009) and indeed, the nAChR-alpha7 receptor is a key element in memory mechanisms (Young et al. 2011; Fig. 5e).

GluR1 receptor complex levels were significantly decreased in trained injected mice when compared with non-injected trained mice that would suggest memory impairment as GluR1 and other AMPA receptors have been shown to be essential for memory mechanisms (Ghafari et al. 2011; Mitsushima et al. 2011). On the other hand, mice lacking the GluR1 showed unimpaired performance in the Morris Water Maze and the results herein are therefore not contradictory to existing concepts (Sanderson et al. 2008). Likewise, yoked injected and yoked non-injected differed significantly in GluR1 complex levels pointing to an IP mediated decline. A training effect on receptor levels was shown as given in Fig. 5f.

The NK-1 receptor complex levels were regulated by IP as shown in Fig. 5g: Memory training per se showed an effect on the NK-1 complex levels in injected and non-injected mice and the IP effect was demonstrated in the yoked groups as well.

NK-1 is important for memory mechanisms and intraperitoneal administration of antagonists show significant effects on memory performance (Kart-Teke et al. 2007).

Decreased NK-1 levels in trained injected mice, however, could be considered as promnestic and are therefore not in disagreement with literature.

Correlation statistics did not reveal significances between receptor levels.

Conclusion

Taken together, several brain receptor levels have been shown to be modified by intraperitoneal injection that in turn may reflect pain. As pain is known to modulate spatial learning and memory (Sandin et al. 2004; Hu et al. 2010) and spatial memory involves the hippocampus, receptor level changes in this brain formation may be of interest. While the biological and pharmacological relevance of the findings herein is still elusive, it is opening a wide area for studies on pain, brain receptors and the hippocampus. It is also intriguing to show that a series of intraperitoneal injections leading to moderate pain is able to modulate hippocampal receptor levels of the muscarinic, nicotinergic, glutamatergic and neurokinin systems. In addition to receptor agonist/antagonist studies it would be of highest interest to examine pain in hippocampal lesionings and mice genetically manipulated for brain receptors. In addition, the concerted action and pattern of hippocampal brain receptor levels following moderate pain by repeated intraperitoneal injections have been addressed. Altered brain receptor complex levels, however, were not over-riding learning and memory performance in the MWM.

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