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PROPAGATION OF THE PSEUDORABIES VIRUS PARTICLES TO THE AMYGDALOID COMPLEX AFTER INOCULATION INTO RAT'S INSULAR CORTEX

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Amygdaloid complex (AC) is a heterogeneous group of subcortical nuclei and cortical structures which has a crucial role in the limbic system functions, and which is interconnected with many brain regions. The projections from the AC to the posterior insular cortex, were studied in 5 male adult rats by retrograde marking of neurons with pseudorabies virus (PRV). After inoculation of PRV into the insular cortex, rats were sacrificed after 51, 57 or 72 hours. The serial sections of AC were examined by using immunohistochemistry, with an antibody against PRV. The goal of this study was to identify the nuclei of rat AC with the presence of PRV, to estimate the density of PRV, and also to note the speed of PRV propagation from the posterior insular cortex to AC.

Clear distribution of PRV particles in amygdaloid nucleus was observed in the rat brain after 72 hours while in earlier sacrificed animals (51 and 57 hours) there was no positive reaction. The high density of PRV-immunoreactive neurons was in the ipsilateral lateral and basolateral nucleus, as well as in the amygdalohippocampal area. The lower density was in the ipsilateral basomedial, central, medial, posterior cortical nucleus, and nuclei of lateral olfactory tracts, as well as in the contralateral basomedial nucleus. In conclusion, by PRV retrograde tracing was found that the main projections of AC to posterior insular cortex originate from lateral and basolateral nucleus which are known as important regions of sensory convergence of AC, and from the amygdalohyppocampal area, as well.

Key words: amygdaloid nuclei, insular cortex, pseudorabies virus, transneuronal tracing, rat

INTRODUCTION

Amygdaloid complex (AC), a heterogenous group of subcortical nuclei and cortical structures, is located in the anteromedial part of the temporal lobe. According to cytoarchitectonic and immunohistochemical research in the AC of the rat can be recognized 13 nuclei and cortical areas and has wide connections with

various brain regions (Krettek and Price, 1978; Ottersen, 1982; Cassell *et al.,* 1982; Moga *et al*., 1989; Canteras *et al*., 1992; McDonald and Mascagni, 1997; Aggleton, 2000).

Many different experiments, including those with ablation or stimulation, showed that the AC is one of the crucial components of the limbic system, especially in the control and modulation of endocrine and autonomic components (visceral effectors mechanisms) of a species-specific complex behavior such as defense, eating, aggression, affects, reproduction, memory and emotions (Kling and Brothers, 1992; Parent, 1995; Cahill and McGaugh, 1998; Aggleton, 2000; Dolan, 2002).

However, the AC nuclei are not separate from one another, but are connected by numerous inner links and they function together (Price *et al*., 1987; Savander *et al*., 1995; Savander *et al*., 1996; Savander *et al*., 1997a,b; Aggleton, 2000; Pitkanen *et al*., 2000). The complex and wide network of significant connections of AC requires extensive studies by the use of different methods.

The recently discovered method of viral transynaptic marking is important in the analysis of neural connections. This technique is based on a capability of neurotropic viruses to infect the chains of hierarchically connected neurons through their synaptic contacts (Aston-Jones and Card, 2000). After inoculation of retrograde transneuronal tracer-pseudorabies virus (PRV) into the rat's insular cortex, the goal of our study was to identify the nuclei of rat AC with the presence of PRV, to estimate the density of PRV particles, and also to observe the speed of PRV propagation from the posterior insular cortex to the AC. We choose IC to be the target for viral application because of its well known involvement in visceral reactions, stress, learning and memory processes (Bermudez-Rantoni et McGaugh, 1991; Saper, 1982), thus being similar and related to the activity of AC.

MATERIALS AND METHODS

All experiments have been done on 5 adult male rats of Sprague-Dawley strain, having a body weight of 250-300 gr. Animals were kept in separate cages (optimal air humidity and temperature of 21-23^{o}C), fed with standard food and free access to water.

In order to investigate the projections from the AC to the posterior insular cortex (IC), we used the method of retrograde marking of the neurons with pseudorabies virus (PRV) in a dose of 80-90 nL per rat. Application of the virus was done under anesthesia with a combination of ketamine (0.2 mL) and 0.2% xylazin-hydrochloride (0.2 mL). After that, the head of the rat was fixed on a stereotaxic table. In order to find and clean the bregma, an incision was performed in the level of the midline suture. Using the Paxinos and Watson (1998) atlas we determined the coordinates of the insular cortex where the virus was applied. The starting point in marking the coordinates on the skull was the bregma. After marking the first two coordinates on the skull, we opened the bone by using a dental drill and took out the dura with a needle. Through the opening the virus was injected with a Hamilton's needle, the skull was closed by the bone which was taken out using gelatin with fibrinogen, and the skin was sutured. Animals were perfused 51, 57 and 72

hours after virus application. Perfusion started with 50 mL of 0.9% NaCl followed by 250-300 mL of Zamboni fixative per rat.

The brains were removed, postfixed in Zamboni fixative overnight at +4°C, and infiltrated with 20% sucrose in 0.1 M phosphate buffer (PBS), pH 7.4 for 24h at +4°C. Coronal sections (50 μm thick) were cut on cryocat (Frigomobile) on -18°C, which is convenient for immunohistohemical reactions on free floating sections. All sections were collected in 0.1M PB with Na-azid until immunoreaction.

After washing in 0.1 M PB and after treatment by 0.5% Triton X-100 for 1 h, sections were washed again with 0.1 M PB and the procedure was continued by adding 3% H_2O_2 in order to block endogenous peroxidase. After washing, the sections were incubated 1 h in 10% normal goat serum. After washing in 0.1 M PB sections were incubated in the primary antiserum specific for PRV (1:7 000). In the primary serum the sections were left on a mixer for 48 hours at +4°C. After this period and washing in PB, sections were incubated with biothinizied anti-rabbit IgG and therafter treated with Vectastatin Elit ABC-peroxidase Kit (Vector Labs). In the ABC complex the sections were left for 1h, and than were washed in 0.1 M PBS and then in TRIS buffer. Visualisation of immunoreactive places was performed in Nickel-DAB (Ni ammonium sulfate 3,3'-diaminobensidine) for 4 min at room temperature.

Background staining was done with Kernechtrot. Ready slices were covered by DePeX. For checking the right place of PRV application, few sections of the insular cortex were mounted on slices and stained with Hematoxilin – Eosin.

RESULTS

After virus application into the deep layers of the posterior insular cortex above the claustrum (Fig 1), the animals were sacrificed after 51, 57 and 72 hours.

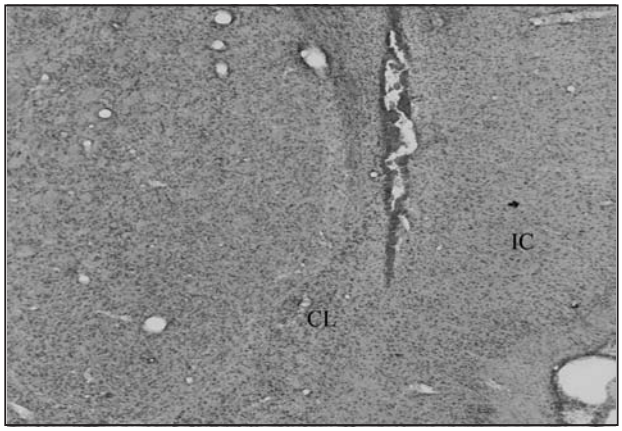

Figure 1. PRV injection site in insular cortex: CL – claustrum, IC – insular cortex. Hematoxilin – Eosin (x 3,2)

The spreading of the PRV through the brain from the insular cortex to AC was investigated. We observed a clear distribution PRV in the rat brain after 72 hours while in earlier sacrificed animals (51 and 57 hours) there was no positive reaction to PRV.

The highest density of PRV-immunoreactive neurons was observed in the ipsilateral lateral (La), basolateral (BL) nucleus and amygdalohyppocampal area (AHA) (Figs. 2 and 3). A lower density of PRV-immunoreactive neurons was in the ipsilateral basomedial (BM), medial (Me), central (Ce) nucleus, nucleus of lateral olfactory tracts (NLOT) and posterior cortical nucleus (CoP), as well as in the contralateral basomedial nucleus (Fig 4).

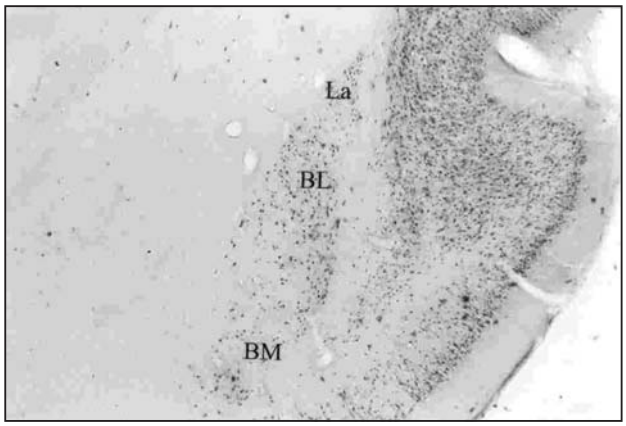

Figure 2. PRV-immunoreactivity in lateral (La), basolateral (BL) and basomedial (BM) rat amygdaloid nuclei (x 3, 2)

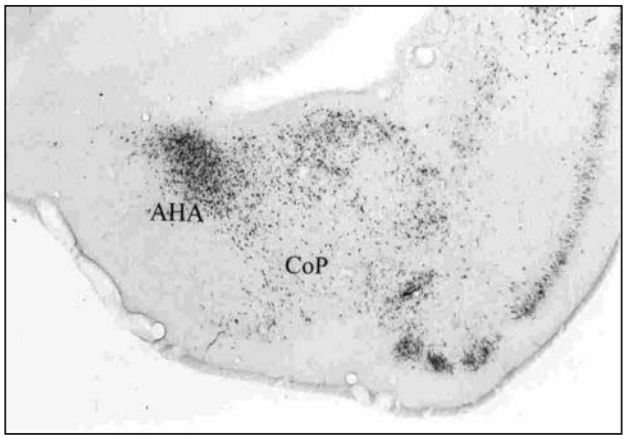

Figure 3. PRV-imunoreactivity in the amygdalo-hippocampal area (AHA) and in the posterior cortical nucleus (CoP) (x 3, 2)

In general, 72 hours after inoculation into rat insular cortex, PRV marked mainly the lateral and basolateral nucleus and the amygdalohyppocampal region.

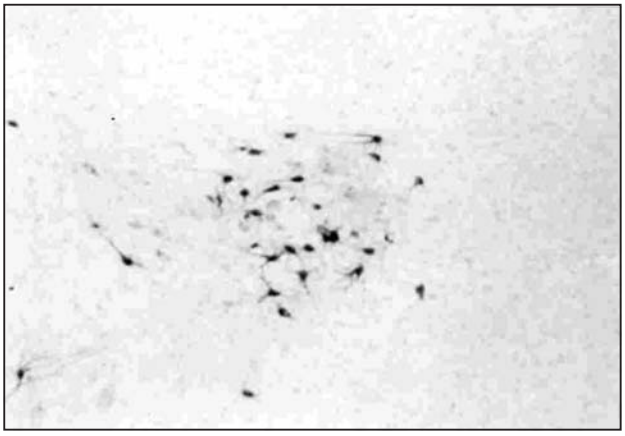

Figure 4. PRV-imunoreactivity in contralateral basomedial amygdaloid nucleus (BM) (x 12, 5)

DISCUSSION

PRV has been used to retrogradely identify spinal cord and brainstem connections to various peripheral organs, but there are no many anatomical studies which have used CNS inoculation of PRV to investigate intrinsic brain connectivity. Because AC has a huge amount of unidirectional or reciprocal connections with numerous regions of the nervous system, we tried by using PRV as retrograde transneuronal tracer to reinvestigate some of these connections.

Amygdaloid nuclei are different in the number and type of functional systems which they supply. So, the fibers from La mainly terminate in the cortical areas (Pitkanen, 2000). Our finding of connections of La to posterior IC is related to the topographically organizied projections terminating in the ventral and dorsal perirhinal cortex and in the posterior agranular insular cortex (Saper, 1982; McDonald and Jackson, 1987). A similar distribution of projections is described for the BL (Saper, 1982; McDonald and Jackson, 1987) and is in agreement with our finding of distribution of PRV particles in BL after inoculation into IC. Interesting is that La and BL are amygdaloid nuclei with the largest density of cocaine- and amphetamine- regulated transcripts (CART) among all AC nuclei of the rat (Puškaš *et al*., 2005). CART has many physiological roles including anxiogenic-like activity in mice and rats (Kask *et al*., 2000) that could have influenced the activity of the insular cortex during stress. It is known that La and BL receive fibers from IC i.e. they are connected bidirectionaly especially with the posterior part (Ottersen, 1982). Thanks to these connections IC is closely involved with the limbic system associated spatial learning (Nerad *et al*., 1996), indicated the fact that

high-frequency stimulation of BL induces long-term potentiation at synapses in the IC (Escobar *et al*., 1998).

Using the PRV as a retrograde tracer we found that both ipsilateral and contralateral BM, project into the insular cortex (IC). Most significant projections from BM are directed into the posterior agranular insular cortex and perirhinal cortex (Saper, 1982; McDonald and Jackson, 1987; Petrovich *et al*., 1996).

We found PRV labelled cells in AAA, as well as in small numbers in Me, Ce, LOT and CoP. Weak projections from Me into the posterior agranular insula were described earlier (McDonald and Jackson, 1987), but Petrovich and Swanson (1997) showed that Ce projects in a small number of brain regions and that there are no projections to the insular cortex or other cortical areas. This difference in distribution of projections could be caused by using different tracers. There are no published data about using PRV in investigations of projections from the Ce. Fibers from the cortical nucleus of AC to other cortical areas are sparse and directed mainly to the posterior agranular insula and to the perirhinal cortex. CoP sends few fibers mainly into the rostral and caudal posterior agranular insula and in the ventral perirhinal cortex (Aggleton, 2000). Our results of LOT projections are in agreement with earlier results on its connections with the posterior insular and with the rostral perirhinal cortex (Aggleton, 2000).

Different to previous results, we found a contingent of labelled cells in AHA. Projections from AHA to the lateral cortical regions are very rare and they mainly originate from the lateral part of AHA and are oriented into the posterior agranular insula and perirhinal cortex (Parent, 1995). This difference in the density of projections in our study could be caused by using PRV as the retrograde tracer or due to the different coordinates.

The ability to infect the chain of hierarchically connected neurons, and the ability to replicate in target cells, i.e. transformation into so called self-replicating markers are the two basic reasons why viruses are considered as ideal transneuronal tracers (Loewy, 1998). However, viruses as neurotropic markers are not universal for all species and types of synapses. So, PRV is highly efficient in the brain of rat and mice and HSV1 is a convenient tracer in the brain of primates (Loewy, 1998). By intracerebral application of the virus there is a possibility of tracer uptake by passing axons. This being negligible for PRV thus making it the tracer which will find wider use in the future (Chen *et al.*, 1999).

Affinity of PRV to neuropil and the concentration of virus in the injection site are extremely important for a successful diffusion, which later leads to productive replication of the virus. The velocity of virus transport and the time necessary for the virus to pass one synapse depend on PRV titer, affinity of neurons and surrounding glia in the injection site, injection volume, and also on the type of synapse. This interval is between 44 to 96 hours. According to our results, PRV 72 hours after inoculation marked mainly projections of the lateral and basolateral amygdaloid nuclei and from amygdalohyppocampal region into the anterior insular cortex. Longer follow up of viral spreading is not possible because the animal dies usually on the fourth day after virus inoculation. Some data suggest that the replication cycle of PRV in the central nervous system lasts nearly six hours (Aston-Jones and Card, 2000). Analysis of PRV replication in astroglia indicates

that in the process of replication exist certain defects which protect these cells from the production of infective offspring.

CONCLUSIONS

The best results in PRV retrograde tracing to AC were obtained 72 hours after virus application into the insular cortex. Lateral, basolateral nuclei and amygdalo-hippocampal area are the main efferent regions of the rat AC to the insular cortex, and in a lesser degree are the medial, central, posterior cortical nucleus and nucleus of lateral olfactory tract.

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ŠIRENJE PSEUDORABIES VIRUSA U AMIGDALOIDNOM KOMPLEKSU PACOVA NAKON INOKULACIJE U INSULARNU KORU

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SADRŽAJ

Amigdaloidni kompleks (AK) je heterogena grupa subkortikalnih jedara i kortikalnih struktura koje imaju važnu ulogu u funkcionisanju limbičkog sistema pomoću brojnih veza koje ostvaruju sa različitim moždanim regijama. U radu su

analizirane veze AK sa zadnjom insularnom korom pomoću retrogradnog obeležavanja neurona modifikovanim virusom besnila (pseudorabies virus – PRV). Nakon inokulacije virusa u insularnu koru, pacovi su žrtvovani nakon 51, 57 ili 72 sata. Serijski preseci mozga u nivou AK su tretirani antitelima na kapsulu virusa imunohistohemijskom metodom. Cilj je bio da identifikujemo jedra AK u kojima je prisutan virus, da utvrdimo gustinu PRV i brzinu propagacije virusa iz zadnje insularne kore u AK.

Jasna distribucija PRV utvrđena je kod životinja žrtvovanih nakon 72 sata, dok kod ranije žrtvovanih životinja nije bilo pozitivne imune reakcije. Veća gustina PRV-imunoreaktivnih neurona utvrđena je u lateralnom i bazolateralnom jedru, kao i u amigdalo-hipokampalnoj oblasti, dok je manja gustina imunoreaktivnih neurona primećena u bazomedijalnom, centralnom, medijalnom, zadnjem kortikalnom jedru, jedru lateralnog olfaktivnog trakta, kao i u kontralateralnom bazomedijalnom jedru. Može se zaključiti da glavne projekcije iz AK polaze iz lateralnog i bazolateralnog jedra koja predstavljaju važno sedište senzorne konvergencije u AK, kao i iz amigdalo-hipokampalne oblasti.